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MECHANISM OF THE THYMOL TURBIDITY TEST

PHILIP P. COHEN, M.D., AND FRANCES L. THOMPSON, B.S.
MADISON, WIS.

THE thymol turbidity test of Maclagan has been shown to be of considerable diagnostic value in certain types of liver disease.[1-4] Maclagan[1] and Carter and Maclagan[4] have reported that the test was probably associated with an increase in gamma globulin. Recant and associates,[5] on the other hand, have shown that the test depended on the presence of a lipid-containing fraction of serum globulin rather than the gamma globulin.

In the present study experiments are reported which show that the thymol turbidity test depends on a reaction involving chiefly the beta globulin fraction of serum.

METHODS AND PROCEDURES

Thymol Reagent and Test.—The thymol reagent was prepared essentially as recommended by Maclagan.[1] It was found that the reagent developed a turbidity on standing after seven to ten days. However, reheating, cooling, and again seeding with thymol crystals resulted in a clear solution.

Thymol turbidity values were estimated from a set of Kingsbury[6] visual standards calibrated against known amounts of human serum albumin and sulfosalicylic acid.

Treatment of Serum With Thymol Reagent for Electrophoretic Analysis.—An aliquot of serum, usually 10 c.c., was diluted with two parts of thymol buffer, pH 7.8, and dialyzed in the cold room (2° C.) against 200 to 250 c.c. of thymol buffer, pH 7.8, for twelve hours or longer. At the end of the dialysis period the precipitate was removed by centrifugation.

The crystal clear supernatant (thymol-treated serum is consistently more translucent than untreated serum) was then dialyzed in the cold room twice against 200 c.c. of 0.1 μ sodium diethylbarbiturate buffer, pH 8.6, and finally against 1,500 c.c. of the same buffer for at least thirty-six hours. The precipitate was resuspended in a volume of 0.1 μ barbiturate buffer, pH 8.6, to give a protein concentration of between 0.5 and 1.0 per cent. The resulting turbid solution was dialyzed in the cold room against several changes of buffer and finally against 1,000 c.c. of buffer for at least thirty-six hours. High speed centrifugation (10,000 r.p.m.) for ten minutes was found effective in clarifying the solution.

Electrophoretic Analysis.—Electrophoresis was carried out at a potential gradient of about 6 volts per centimeter, at 1.5° C., using the schlieren scanning

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technique of Longsworth.[7] Most of the runs were made with the single center section 11 c.c. cell. A micro cell, 2 c.c. capacity, was employed in some instances for both the thymol precipitate and the treated and untreated serum samples. A comparative study of the patterns and mobilities of the same serum with the macro and micro cells revealed close agreement in a series of ten determinations.

Relative composition of the serum samples was estimated by measuring the areas under the projected patterns after the areas were resolved into a series of symmetrical curves. In all instances the percentage composition represents the average of the ascending and descending boundaries. Mobilities were de-

TABLE I. ELECTROPHORETIC COMPOSITION OF SERUM BEFORE AND AFTER TREATMENT WITH THYMOL REAGENT

SERUM	DIAGNOSIS	THYMOL TURBIDITY UNITS	TOTAL PROTEIN (GM./100 C.C.)	PER CENT COMPOSITION				
				ALBUMIN	ALPHA-1	ALPHA-2	BETA	GAMMA
1A*	Laënnec's cirrhosis	4	5.8	32.6	7.3	7.6	17.0	35.5
1B*				41.1	7.0	12.3	7.5	32.1
2A	Infectious hepatitis	10	7.1	40.7	6.2	12.9	19.0	21.2
2B				49.6	7.4	15.0	8.5	19.5
3A	Infectious hepatitis	9	7.2	45.5	4.5	9.7	18.4	21.9
3B				51.8	5.0	10.6	12.6	20.0
4A	Infectious hepatitis	14	8.1	39.8	5.6	10.6	16.4	27.6
4B				40.8	5.7	8.9	13.3	31.3
5A	Biliary cirrhosis	8	6.0	27.3	4.7	8.2	15.0	44.8
5B				34.5	4.6	9.2	8.0	43.7
6A	Infectious hepatitis	10	7.8	43.7	4.9	9.0	17.9	24.5
6B				54.7	5.6	10.5	6.5	22.7
7A	Infectious hepatitis	6	8.3	40.0	5.4	10.6	15.6	28.4
7B				45.1	5.9	10.9	9.2	28.9
8A	Chronic hepatitis	4	7.2	51.3	3.8	8.4	18.2	18.3
8B				58.5	5.0	8.4	12.4	15.7
9A	Infectious hepatitis	4	6.8	51.8	4.6	10.0	19.9	13.7
9B				55.7	6.3	10.4	12.4	15.2
10A	Infectious hepatitis	8	7.2	53.6	4.2	8.6	16.2	17.4
10B				60.3	5.0	12.3	7.6	14.8
11A	Chronic hepatitis;	10	5.0	43.6	5.8	10.0	15.9	24.7
11B	renal tuberculosis			50.3	6.9	10.9	7.7	24.2
11C*				50.7	8.0	12.3	6.3	24.0
	Average A			42.7	5.2	9.6	17.2	25.3
	Average B			48.4	5.7	10.9	9.6	24.4
12A	Normal	0	7.1	56.7	6.8	9.0	13.2	14.3
12B				59.0	6.2	9.2	11.7	13.9
12C				58.1	8.6	8.9	10.2	14.2
	Electrophoretic Analysis of Ten Sera From Normal Adults							
Mean				56.5	5.2	9.0	15.2	14.2
Standard deviation				2.8	1.7	1.4	1.5	1.8
Standard error of mean				0.9	0.6	0.5	0.5	0.6

*Sample A refers to untreated serum; sample B refers to serum after dialysis for twelve hours against thymol reagent and removal of precipitate; sample C refers to serum after dialysis for seventy-two hours against thymol buffer and removal of precipitate.

termined by measuring the migration distances from the salt boundaries. Conductivity measurements were made on the protein-free buffer used as the dialyzing solution.

RESULTS

The percentage composition of serum before and after thymol treatment is shown in Table I. In each instance the thymol turbidity value is indicated. The most striking change in the composition of the serum after treatment with thymol reagent is the decrease in the beta globulin fraction. As can be seen,

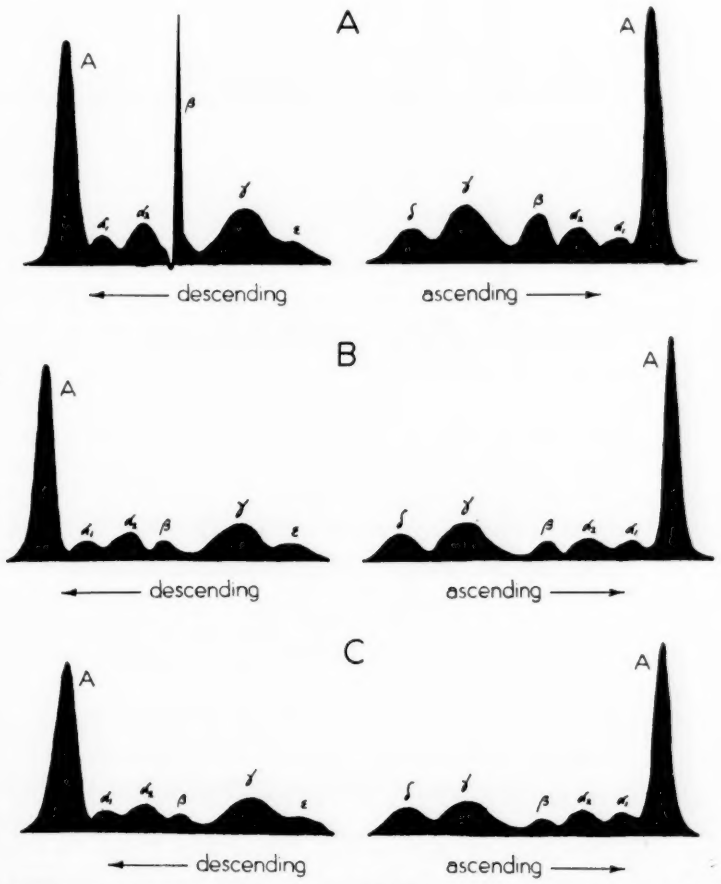

Fig. 1.—Electrophoretic diagrams of Serum 11 (Table I) before treatment with thymol reagent (*A*), after treatment for twelve hours (*B*), and after treatment for seventy-hours (*C*).

this fraction is decreased in all instances including the normal serum although the decrease in the latter case is small. Treatment of a thymol-negative and a thymol-positive serum for seventy-two hours instead of the usual twelve hours with thymol reagent resulted in a small additional decrease in the beta globulin percentage (Serum 11C and 12C, Table I). The average decrease in the beta globulin percentage as a result of thymol treatment is of the order of 50 per

cent. The average decrease in the percentage of gamma globulin is of the order of 4 per cent. All the other fractions show an increase, the albumin percentage being increased the most. It is apparent from these data that the beta globulin fraction is the only fraction of serum showing a striking change as a result of thymol treatment. The electrophoretic diagrams of Serum 11 (Table I) before and after treatment with thymol reagent are seen in Fig. 1. The disappearance of the beta anomaly after thymol was seen in all instances.

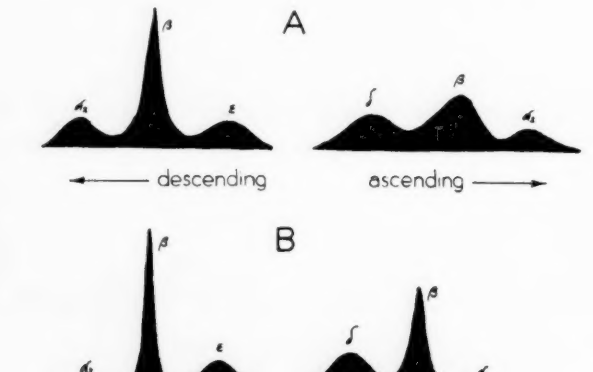

Fig. 2.—Electrophoretic diagrams of thymol precipitates. *A*, From normal (Serum 12, Table I); *B*, chronic hepatitis (Serum 11, Table I); *C*, mixture of precipitates *A* and *B*.

Further evidence in support of the role of beta globulin is seen in the electrophoretic diagrams of the thymol precipitates (Fig. 2). Diagram *A* of Fig. 2 is that of the thymol precipitate from normal Serum 12 (Table I); diagram *B* is that from Serum 11 (Table I); diagram *C* represents an equal mixture of thymol precipitate from Sera 11 and 12 (Table I). From the diagrams it is apparent that the thymol precipitate consists of one major component which has a mobility of a beta globulin (Table II) and a smaller and irregularly appearing component which has a mobility of an alpha-2 globulin. It is of interest to note that the descending patterns of the thymol precipitates have the appearance of a beta anomaly. When the thymol precipitates of a normal and pathologic serum are mixed (diagram *C*, Fig. 2), the components have the same mobility and composition as the unmixed precipitates. The mobilities of the various components of serum before and after thymol treatment, of the precipitates, and of normal serum are shown in Table II.

TABLE II. MOBILITIES (CM.²/VOLT SEC. × 10^{-5})

	ALBUMIN	ALPHA-1	ALPHA-2	BETA	GAMMA
Normal					
Mean	6.92	5.90	4.70	3.42	1.64
S.D.	0.23	0.25	0.20	0.28	0.29
Liver disease					
Before thymol					
Mean	6.95	6.1	4.86	3.63	1.70
S.D.	0.35	0.32	0.22	0.25	0.27
After thymol					
Mean	6.95	6.0	4.92	3.75	1.72
S.D.	0.31	0.30	0.31	0.33	0.32
Thymol precipitate					
Mean	—	—	—	3.30	—
S.D.	—	—	—	0.32	—

DISCUSSION

It is obvious from the data presented that thymol reagent reacts chiefly if not exclusively with the beta globulin fraction of serum. The fact that the beta globulin levels in the thymol turbidity-positive cases are not strikingly higher than those observed with a series of normals (Table I) suggests that this fraction has an abnormal component. This is further supported by the fact that treatment of normal serum with thymol reagent results in only a small decrease in the beta globulin level (Serum 12, Table I). The precipitate, however, is indistinguishable, electrophoretically, from that obtained from pathologic serum (diagrams *A* and *C*, Fig. 2).

It is of some interest to compare the electrophoretic patterns of sera of this series of liver diseases with those from normal adults. If the average values of the pathologic sera are compared with the average values of the normal series (Table I), it will be noted that in the former group the albumin is decreased, the beta globulin is increased slightly, and the gamma globulin is strikingly increased. It should be noted also that while the gamma globulin level is increased above the normal value in all cases but Serum 9, there is no correlation between the gamma globulin increase and the thymol turbidity units. A similar lack of correlation, however, is also to be seen in the case of the beta globulins.

The known complexity of the beta globulin fraction makes any attempt at a discussion of the possible nature of the changes in this fraction in disease difficult. It is possible that there is no fundamental change in the protein components making up this fraction, but rather a change in either the kind or amount of phospholipid chemically bound with the protein. While most patients with nonobstructive liver disease do not show any consistent increase in phospholipid content of serum,[10] it is possible that some alteration in the amount or type of true lipoprotein rather than total lipid is involved.

A sample of the thymol precipitate from Serum 11 was examined in the ultracentrifuge. The material had the same number and kind of components as are seen with the beta globulin fraction obtained as a by-product of fractionation of gamma globulin from normal human plasma.[8] This normal beta globulin fraction was approximately 90 per cent homogeneous electrophoretically.[9] Thus it would appear that on the basis of the properties in the electrophoresis

apparatus and in the ultracentrifuge, there are no physical differences to be noted between the normal and pathologic beta globulin fractions.

SUMMARY

1. Electrophoretic analyses of sera from patients with liver disease having positive thymol turbidity tests were made before and after treatment with thymol reagent. The chief change noted was a decrease of the beta globulin fraction of the supernatant after removal of the thymol precipitate.

2. The thymol precipitates obtained from sera from both normal and diseased cases were examined in the electrophoresis apparatus and both were shown to migrate with the mobility of a beta globulin. Examination in the ultracentrifuge revealed the thymol precipitate to have a composition similar to that seen with the beta globulin fractions obtained from normal human plasma.

3. The possible basis for the thymol turbidity test is briefly discussed.

REFERENCES

1. Maclagan, N. F.: The Thymol Turbidity Test as an Indicator of Liver Dysfunction, Brit. J. Exper. Path. **25**: 234, 1944.
2. Shank, R. E., and Hoagland, C. L.: A Modified Method for the Quantitative Determination of the Thymol Turbidity Reaction of Serum, J. Biol. Chem. **162**: 133, 1946.
3. Watson, C. T., and Rappaport, E. M.: A Comparison of the Results Obtained With the Hanger Cephalin-Cholesterol Flocculation Test and the Maclagan Thymol Turbidity Test in Patients With Liver Disease, J. Lab. & Clin. Med. **30**: 983, 1945.
4. Carter, A. B., and Maclagan, N. F.: Some Observations on Liver Function Tests in Diseases Not Primarily Hepatic, Brit. M. J. **2**: 80, 1946.
5. Recant, L., Chargaff, E., and Hanger, F. M.: Comparison of the Cephalin-Cholesterol Flocculation With the Thymol Turbidity Test, Proc. Soc. Exper. Biol. & Med. **60**: 245, 1945.
6. Kingsbury, F. B., Clark, C. P., Williams, G., and Post, A. L.: The Rapid Determination of Albumin in Urine, J. Lab. & Clin. Med. **11**: 981, 1926.
7. Longsworth, L. G.: Recent Advances in the Study of Proteins by Electrophoresis, Chem. Rev. **30**: 323, 1942.
8. Deutsch, H. F., Alberty, R. A., and Gosting, L. J.: Biophysical Studies of Blood Plasma Proteins. IV. Separation and Purification of a New Globulin From Normal Human Plasma, J. Biol. Chem. **165**: 21, 1946.
9. Deutsch, H. F.: Personal communication.
10. Man, E. B., Kartin, B. L., Durlacher, S. H., and Peters, J. P.: The Lipids of Serum and Liver in Patients With Hepatic Diseases, J. Clin. Investigation **24**: 623, 1945.

A STUDY OF THE SERUM BILIVERDIN CONCENTRATION IN VARIOUS TYPES OF JAUNDICE

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THE presence of a green tinge in the skin of certain patients with jaundice has long been recognized. The relation of this so-called biliverdin jaundice to the underlying disease has been discussed by various writers.[6, 7, 10, 14]

According to recent chemical evidence,[2, 3, 11, 18-23, 31] it is probable that biliverdin is intermediate in the catabolism of hemoglobin to bilirubin. Demonstration by Rich[27, 28] of both biliverdin and bilirubin in the phagocytes of tissue cultures of mesothelial origin, the occurrence of biliverdin in the Kupffer cells of geese as observed by McNee,[25] and its presence in dog's placenta[18] and hematoma fluid[16] emphasize a close physiologic connection between biliverdin and hemoglobin breakdown. The "green hemoglobins" studied by Fischer,[11] Warburg,[31] Lemberg,[18-23] Barkan,[2, 3] and their co-workers, to which the name "verdohemochromogen" was given by Lemberg,[20] are undoubtedly biliverdin-iron-protein complexes. Assuming that biliverdin is an intermediate stage in the transition of hemoglobin to bilirubin, its reduction to the latter substance in the body normally proceeds in a manner that excludes its appearance in the serum or plasma in evident amounts.

Methods have been described for the determination of biliverdin in bile,[1, 12] but these are not suitable for blood serum or plasma. Biliverdin does not give a diazo reaction and, thus, is not included in the quantitative van den Bergh method for bilirubin, regardless of modification. Specific color reactions for biliverdin are unknown. The purpose of the present study was to develop a method for the quantitative determination of biliverdin in serum or plasma and to apply this method in studying various patients with jaundice, especially those exhibiting greenish or outspoken green tints.

METHODS

Crystalline biliverdin was prepared by the addition of purified bilirubin* to boiling glacial acetic acid containing ferric chloride, according to Lemberg's method.[17] The dry crystalline substance was weighed on a microbalance, and a 0.4 mg. per cent solution in 0.01 per cent sodium hydroxide was prepared. Employing the Evelyn photoelectric colorimeter, a transmission curve then was determined for this solution. A like curve was obtained for a similarily prepared solution of bilirubin. These curves are shown in Fig. 1. Inspection establishes the suitable wave length for measurement of biliverdin between 635 and 690 millimicrons. Between these two points bilirubin transmits well, whereas biliverdin has a relatively great absorption. Thus, the 660 mμ filter was employed in the present study.

From the Department of Internal Medicine and Hospital Laboratories of the University of Minnesota Hospitals.
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*Obtained through the courtesy of Dr. J. D. Porsche of Armour and Company, Chicago, Ill.

The use of direct regional spectrophotometry in this determination necessitates exclusion of the presence of appreciable quantities of reduced and oxyhemoglobin, methemoglobin, methemalbumin, hematin, and sulfhemoglobin. These interfering pigments, in contrast to biliverdin, absorb more light in the region of 620 to 635 mμ than at 660; initial comparison in these two regions thereby serves to indicate their presence. Fortunately the interfering pigments are seldom encountered in carefully manipulated unhemolyzed specimens, though the presence of any appreciable quantity of any one substance casts doubt upon the results of the biliverdin determination. Furthermore, the use of nonturbid, fasting sera is obviously necessary. The reader is referred to the work of Drabkin and Austin[8] for further orientation concerning the light absorption of the various forms of hemoglobin.

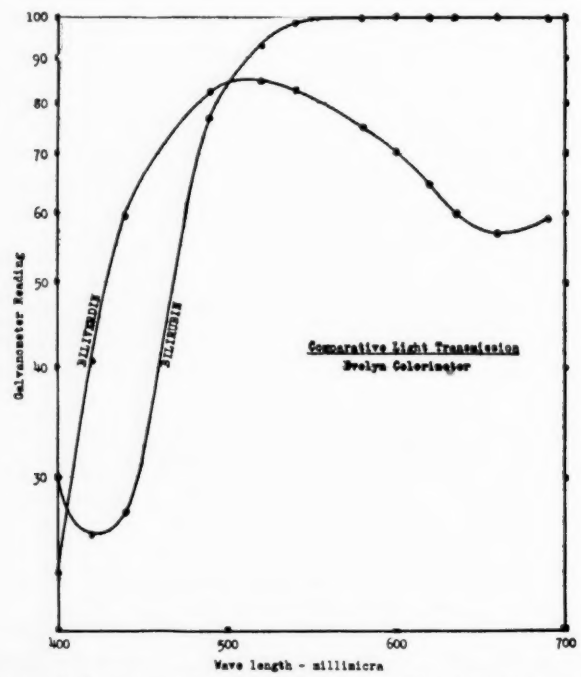

Fig. 1.—Transmission curves for solutions of crystalline bilirubin and biliverdin.

A calibration curve was determined as follows: One cubic centimeter quantities of clear, fasting normal sera, containing varying known quantities of added crystalline biliverdin in solution, were added to 8 c.c. quantities of distilled water and mixed. The resultant turbidity was then dispelled by the addition of 1 c.c. of saturated sodium chloride solution. Utilizing the Evelyn colorimeter, the 660 mμ filter, and a predetermined center setting (see next paragraph), a straight-line relationship was observed when the galvanometer readings were plotted against concentration on semilogarithmic paper (Fig. 2).

Normal sera, containing no biliverdin as evidenced by no dip in transmission between 620 and 660 mμ, even when they appear to be clear, may give

galvanometer readings between 98 and 100 when a blank (containing 9 c.c. of water and 1 c.c. of saturated sodium chloride) is set at 100. Consequently, a center setting of the instrument is determined from a diluted normal serum which, after giving a reading of 98 against the theoretical blank, is then set to read 100. This precaution prevents the apparent measurement of biliverdin when actually none is present, but conversely, very small amounts, less than 0.02 mg. per cent may be missed.

The serum bilirubin was determined by the method of Malloy and Evelyn,[24] modified in accordance with the recommendation of Ducci and Watson.[9]

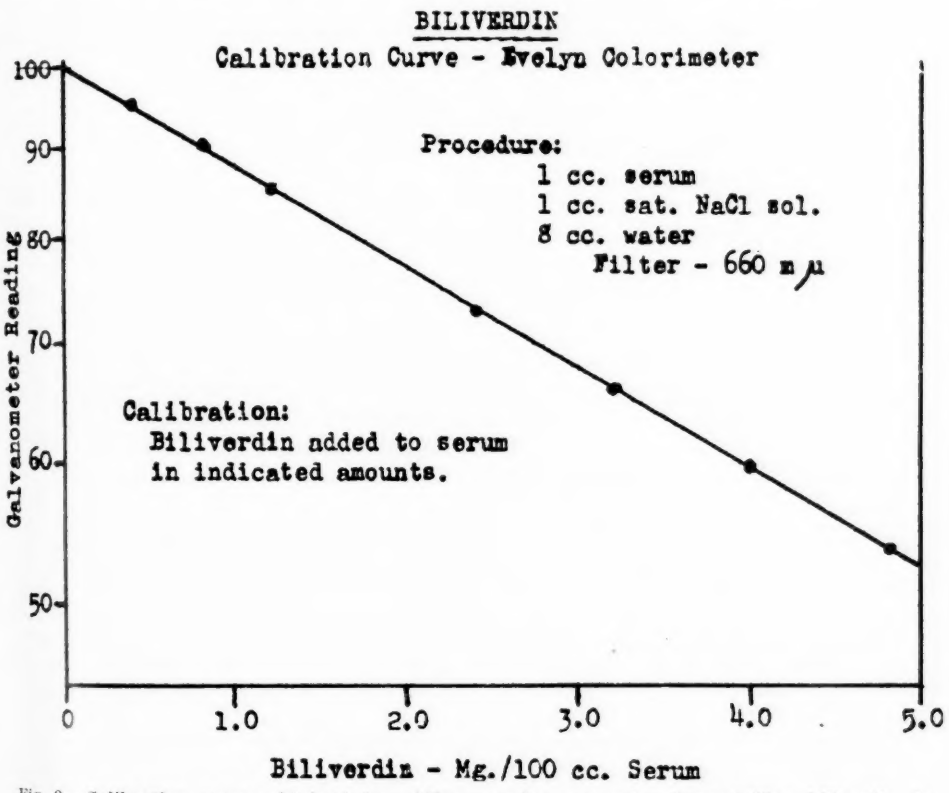

Fig. 2.—Calibration curve obtained by adding varying amounts of crystalline biliverdin to normal serum.

RESULTS

The sera of twelve nonjaundiced individuals, including both hospital patients and normal healthy persons, were examined for biliverdin. None was found in this series by the method described. Values ranging from zero to 2.2 mg per cent were obtained in the course of 105 observations on the sera of sixty-six patients with jaundice.

Significant levels of serum biliverdin were found in the following patients: twenty-seven with carcinomatous obstruction of the common bile duct (Fig. 3), five with infectious hepatitis (Fig. 4), five with cirrhosis and jaundice

TABLE I. PATIENTS WITH JAUNDICE WITH NO BILIVERDIN IN SERUM

PATIENTS	CONDITION	BILIRUBIN DIRECT TOTAL	BILIRUBIN TOTAL MG. %
6	Transfusion reaction	.11	5.4
		.08	4.6
		--	11.0
		--	3.0
		.83	30.0*
		.62	3.4
3	Hemolytic anemia	.17	3.6
		.19	5.0
		.66	14.1
1	Infectious mononucleosis	.78	3.2
1	Myelogenous leucemia	.16	3.3
1	Hodgkin's disease	.22	11.3
1	Banti's disease	.31	9.3
		.52	5.5
1	Cardiac Failure	.42	4.0

*Questionable biliverdin.

(Fig. 4), and five with common bile duct stenosis (Fig. 5). Among these were fourteen individuals with a total bilirubin under 10 mg. per cent (average, 5.9 mg. per cent). On the other hand, of ten with calculous obstruction of the common duct, five had no biliverdin in the serum, although their average total bilirubin was 7.1 mg. per cent (Fig. 5).

The remaining fourteen patients (Table I) with a range of total bilirubin from 3 to 30 mg. per cent (average 7.6 mg. per cent), twelve of whom had

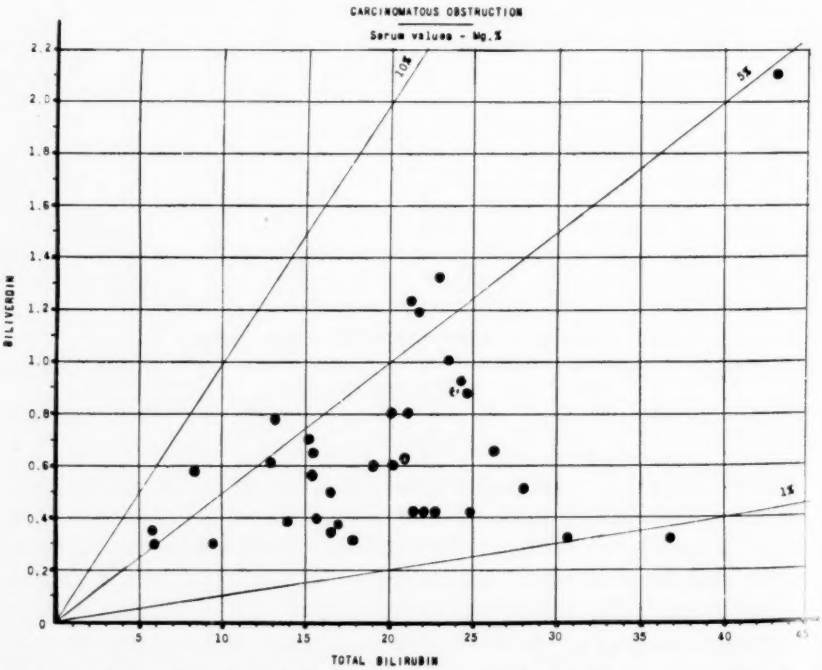

Fig. 3.—Serum biliverdin values in twenty-seven patients with carcinomatous obstruction of the common bile duct plotted against the total serum bilirubin (per cent biliverdin of total bilirubin represented by the radiating lines).

either a hemolytic process or bilirubinemia of the predominantly indirect type, all had zero biliverdin. The interference of methemalbumin in the method was not recognized early in the study but undoubtedly accounts for the slight galvanometer deflection in the patient marked by the asterisk (Table I), in which a definite in vivo hemolysis was known to have occurred.

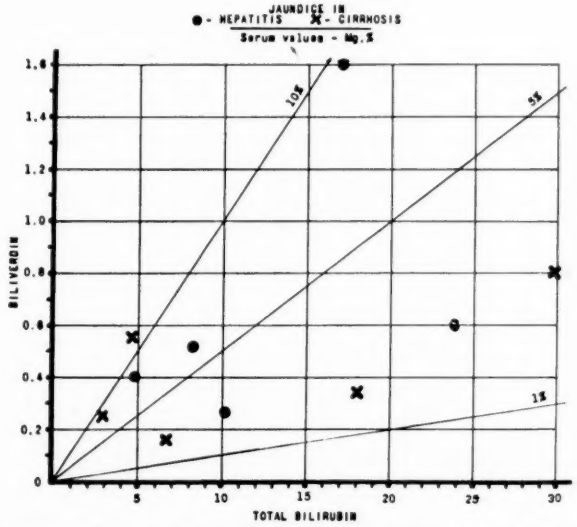

Fig. 4.—Serum biliverdin values plotted against total serum bilirubin in patients with infectious hepatitis and cirrhosis.

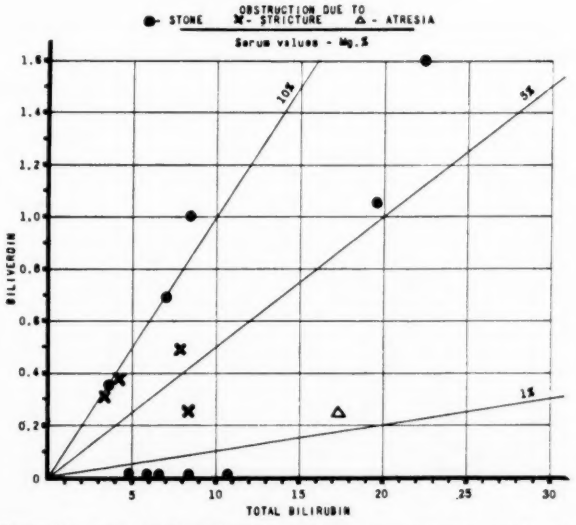

Fig. 5.—Serum biliverdin and total bilirubin values in patients with stone, stricture, and atresia of the common bile duct.

A variation in biliverdin concentration without respect to the direction of change in total bilirubin was noted during serial observations on twelve patients (Figs. 6 and 7); of these, the three in Fig. 7 stood out as being the

only ones who had received food or intravenous feeding to raise their caloric intake above basal requirements, resulting in a weight gain in two instances. In these three biliverdinemia disappeared completely despite the absence of any marked change in the total serum bilirubin.

A green tint was observed in the skin of patients having as little as 0.3 mg. per cent in the serum.

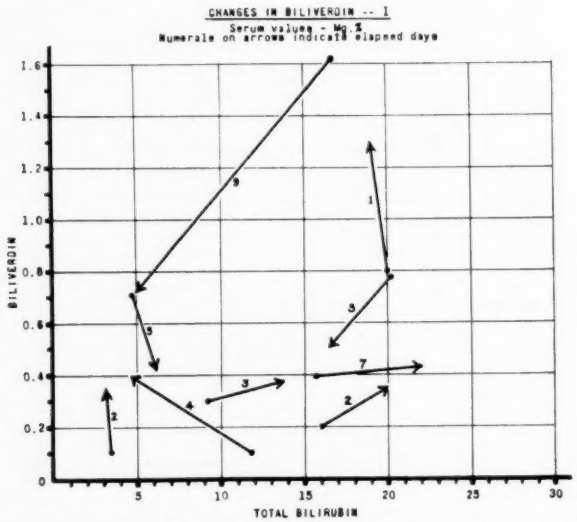

Fig. 6.—Changes in the serum biliverdin and bilirubin concentrations in serial studies.

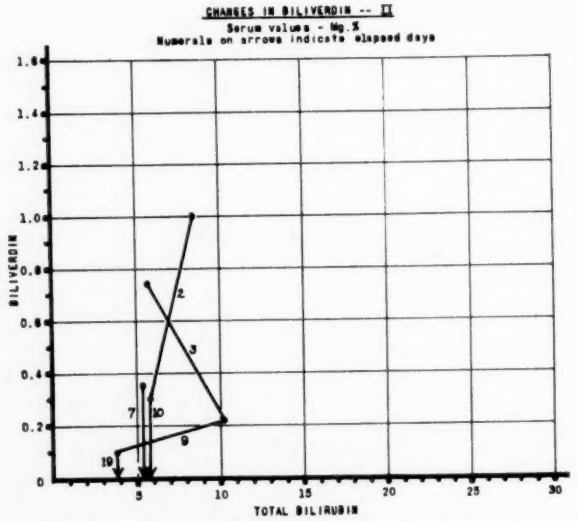

Fig. 7.—Disappearance of biliverdinemia without corresponding change in total serum bilirubin in patients with notably high caloric intakes.

DISCUSSION OF RESULTS

Lemberg and Wyndham[21] demonstrated a rapid, in vitro transformation of biliverdin to bilirubin in the presence of liver, kidney, and brain tissue and a

slower change in the presence of other tissues. They also observed a more rapid and complete transformation by the liver tissue of well-fed animals as compared with that of starved animals; furthermore, the addition of glucose to the tissue preparations from the latter animals accelerated the change. Lemberg[22] further emphasized the role of the liver in the reduction of biliverdin to bilirubin and the particularly close connection between the metabolism of bile pigment and carbohydrate in the liver.

The latter hypothesis gains possible support from the experience of Kanasaki,[15] in which green bile in the duodenal drainage of a patient with cirrhosis was replaced by orange bile after administration of intravenous glucose. Furthermore, biliverdin rather than bilirubin is excreted in the bile of starving dogs[13] and by frogs[26] whose livers are low in glycogen content. The oxidation-reduction possibilities which may exist in the liver and biliary duct system, both in health and disease, have been discussed by a number of investigators.[4, 5, 22, 23, 29, 30]

In the present study the trend was for the higher values of serum biliverdin to occur in patients with the higher values for total bilirubin, though notable exceptions occurred. In agreement with the findings of Eppinger,[10] the results indicate that the most pronounced biliverdin icterus and the highest values for serum biliverdin were associated with neoplastic obstruction of the common bile duct. However, biliverdinemia was a feature of other types of regurgitation jaundice as well, especially infectious hepatitis. The bulk of evidence favors a failure of change in biliverdin to bilirubin in the liver and/or other tissues. Noteworthy in this respect is the lack of biliverdinemia in the patients with hemolytic jaundice in which there was an obvious rapid transformation of blood to bile pigment. As to causes of biliverdinemia other than a failure of the liver or other organs and tissues to reduce biliverdin to bilirubin, the spontaneous oxidative formation of biliverdin from bilirubin in the gall bladder and bile ducts, with subsequent reabsorption, must be considered. In this regard, the patients with a high biliverdinemia did not show greater clinical evidence of infection than those with equal jaundice and less biliverdinemia. Furthermore, serial observations in twelve patients indicated that the serum biliverdin concentration can change markedly in one to five days without corresponding change in total bilirubin and, therefore, in the case of common duct obstruction, without change in the reabsorption possibilities from the biliary tree.

In contrast to the clinical observations of Brugsch[6] and Horsters,[14] biliverdinemia was not observed in jaundice due to hemolytic processes.

SUMMARY AND CONCLUSIONS

1. A direct spectrophotometric method for the quantitation of biliverdin in serum or plasma is described.

2. Observations on sixty-six patients with jaundice due to various causes are summarized. In general, biliverdinemia is a feature of regurgitation jaundice; it has not been observed in hemolytic (retention) jaundice. Absence of biliverdinemia speaks against a diagnosis of jaundice due to neoplastic obstruction.

3. There is suggestive evidence that biliverdinemia is abolished in jaundice when the nutritional status of the patient improves.

REFERENCES

1. Amada, Y.: Versuche über die quantitative Erfassung der Verdinanteile der Gallenfarbstoffe in der Galle, J. Biochem. **32:** 187, 1940.
2. Barkan, G.: Blutfarbstoff, Eisen, Gallenfarbstoff; neuere Untersuchungen über eisenhaltige Begleiter des Hämoglobins, Klin. Wchnschr. **16:** 1265, 1937.
3. Barkan, G., and Schales, O.: Chemischer Aufbau und physiologische Bedeutung des "leicht abspaltbaren" Bluteisens. 13. Mitteilung in der Reihe der Eisenstudien, Ztschr. f. physiol. Chem. **248:** 96, 1937.
4. Barry, H. M., and Levine, V. E.: The Oxidation and Reduction of Bile Pigments, J. Biol. Chem. (Proceedings) **59:** lii, 1924.
5. Baumgärtel, T.: Zur Kenntnis der biologischen Erythrocytolyse, Klin. Wchnschr. 22: 457, 1943.
6. Brugsch, T.: Mechanisch bedingter Ikterus, Med. Klin. **31:** 366, 1935.
7. Davies, D. T., and Dobbs, E. C.: A Study of the Properties of Pure Bilirubin and Its Behavior Toward the van den Bergh Reagent, Brit. J. Exper. Path **8:** 316, 1927.
8. Drabkin, D. L., and Austin, J. H.: Spectrophotometric Studies. II. Preparations From Washed Blood Cells; Nitric Oxide Hemoglobin and Sulfhemoglobin, J. Biol. Chem. **112:** 51, 1935.
9. Ducci, H., and Watson, C. J.: The Quantitative Determination of the Serum Bilirubin With Special Reference to the Prompt-Reacting and the Chloroform-Soluble Types, J. Lab. & Clin. Med. **30:** 293, 1945.
10. Eppinger, H.: Die Leberkrankheiten, Berlin, 1937, p. 246, Julius Springer.
11. Fischer, H., and Lindner, F.: Ueber den Umbau des Blutfarbstoffes durch Hefe, Ztschr. f. Physiol. Chem. **153:** 54, 1926.
12. Hooper, C. W., and Whipple, G. H.: Bile Pigment Metabolism, Am. J. Physiol. **40:** 332, 1916.
13. Hoppe-Seyler: Thierfelder, Handbuch d. physiol. u. pathol-chem. Analyse, Berlin, 1924, Julius Springer, 9 Aufl., p. 417.
14. Horsters, H.: Differentialdiagnose des Ikterus nach färberischen Merkmalen, Ergebn. d. inn. Med. u. Kinderh. **56:** 575, 1939.
15. Kanasaki, K.: The Effect of the Injection of Glucose Upon Green Bile, Jap. J. Gastroenterol. **5:** 91, 1933.
16. Langhans, T.: Beobachtungen über Resorption der Extravasate und Pigmentbildung in denselben, Virchows Arch. **49:** 66, 1870.
17. Lemberg, R.: Ueber Dehydrobilirubin, Liebig's Ann. d. Chem. **499:** 25, 1932.
18. Lemberg, R., and Barcroft, J.: Uteroverdin, the Green Pigment of the Dog's Placenta, Proc. Roy. Soc., London, s.B **110:** 362, 1932.
19. Lemberg, R.: Bile Pigments. VI. Biliverdin, Uteroverdin, and Oocyan, Biochem. J. **28:** 978, 1934.
20. Lemberg, R.: Transformation of Haemins Into Bile Pigments, Biochem. J. **29:** 1322, 1935.
21. Lemberg, R., and Wyndham, R. A.: Reduction of Biliverdin to Bilirubin in Tissues, Biochem. J. **30:** 1147, 1936.
22. Lemberg, R.: The Disintegration of Haemoglobin in the Animal Body, Perspectives in Biochemistry, pp. 137-149, Cambridge University Press, 1937.
23. Lemberg, R., Lockwood, W. H., and Legge, J. W.: Coupled Oxidation of Ascorbic Acid and Hemoglobin. V. Studies on Formation of Bile Pigments From Choleglobin and Verdohaemochromogen and Their Isolation From Erythrocytes, Biochem. J. **35:** 363, 1941.
24. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, J. Biol. Chem. **119:** 481, 1937.
25. McNee, J. W.: Experiments on Haemolytic Icterus, J. Path. & Bact. **18:** 328, 1913.
26. Nisimaru, Y.: Bile Pigment Formation From Hemoglobin, Am. J. Physiol. **97:** 654, 1931.
27. Rich, A. R.: The Formation of Bile Pigment From Haemoglobin in Tissue Cultures, Bull. Johns Hopkins Hosp. **35:** 415, 1924.
28. Rich, A. R.: The Formation of Bile Pigment, Physiol. Rev. **5:** 182, 1925.
29. Schreus, H. T., and Carrié, C.: Untersuchungen zum Gallenfarbstoffwechsel. 1. Mitteilung; Abbau des Blutfarbstoffe zu Protoporphyrin durch Leber und andere Organe; nachweiseiner Fermentwirkung, Klin. Wchnschr. **13:** 1670, 1934.
30. Sumner, J. G., and Nymon, M.: The Oxidation of Bilirubin by Peroxidase, Science **102:** 209, 1945.
31. Warburg, O., and Negelein, E.: Grünes Haemin aus Blut-Haemin, Ber. deutsch. chem. Ges. **63:** 1816, 1930.

DIFFERENTIAL AGGLUTINATION OF HUMAN ERYTHROCYTES

Evaluation of Technique

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THE differential agglutination of erythrocytes depends upon a specific reaction between the agglutinin of an antiserum and the agglutinogen of red cells. After transfusion of red cells lacking an agglutinogen present in the cells of the recipient, it is possible to agglutinate the cells of the recipient by use of an appropriate antiserum. Conversely, after transfusion of erythrocytes containing an agglutinogen not present in the cells of the recipient, but for which the recipient's serum has no specific agglutinin, the donated corpuscles can be differentially agglutinated.

Inasmuch as this procedure has many applications in investigative medicine, it was deemed worth while to study the factors affecting the accuracy of the method and to determine the most satisfactory quantitative procedure. The objectives of this paper are (1) to describe the technique currently used by this laboratory and (2) to present the practical, experimental, and statistical considerations which led to the adoption of this technique.

HISTORICAL

In 1919 Ashby[1] first studied the fate of transfused erythrocytes by giving Group O cells to recipients belonging to one of the other major groups. She found, for example, that after transfusing Group O cells to a Group A recipient, the latter's cells could be agglutinated with anti-A (Group B) serum and that the unagglutinated O cells of the donor could then be enumerated by transferring the serum-cell mixture to a counting chamber. Wiener[2, 3] later demonstrated that differences in MN type could be utilized in a similar manner. After transfusing AN cells, for instance, to an AMN recipient, the latter's cells were agglutinated by anti-M serum and the unagglutinated AN cells of the donor could be counted as in the method of Ashby. By utilizing both ABO and MN differences, it was soon proved feasible to make observations simultaneously on the fate of cells from two or three different donors in the circulation of a single recipient.

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Ashby's technique consisted of filling a leucocyte pipette with capillary blood to the .5 mark. The pipette was then filled to the 1.1 mark with a 20:1 mixture of serum and 4.4 per cent citrate solution. The mixture was expelled into a small test tube, shaken, incubated at 37° C. for forty minutes with shaking every ten minutes, and then placed in an icebox overnight. On the following day the mixture was shaken, a drop placed in a counting chamber, and the cells in 160 small squares in each of two chambers were counted. A tube containing the blood of a nontransfused individual was used as a control to test the agglutinative capacity of the serum, and the same serum was used throughout each experiment. Ashby[4] later advised against refrigeration of serum-cell mixtures because of nonspecific agglutination caused by low temperatures.

In 1922 when Wearn and associates[5] used Ashby's method to determine the life of transfused red cells, they noted that, of the cells which should have been specifically agglutinated, from 20,000 to 50,000 per cubic millimeter remained unagglutinated and they considered 50,000 such cells per cubic millimeter the upper limit for satisfactory sera.

In 1924 Jervell[6] made a comprehensive study of the factors affecting the accuracy of Ashby's technique. He used saline suspensions of cells mixed with agglutinating sera of high titer and found that shaking of serum-cell mixtures for one minute was adequate and that further shaking did not alter the counts.

Landsteiner and co-workers[7] in 1928 first employed M and N factors in a study of the longevity of type M donated cells in a recipient of type N, and Wiener and associates[8, 9] applied M-N differences to the study of transfusion reactions and storage of blood.

Mollison and Young,[10, 11] who applied the principle of differential agglutination to the study of various blood preservatives, drew capillary blood into a hemoglobinometer pipette, mixed it with citrate, and then mixed the cell suspension with antiserum in a flat-bottomed bottle. Anti-A, -B, -M, and -N sera were used, and two individuals enumerated the unagglutinated cells.

Dacie and Mollison[12] in 1943 further refined the technique during the course of their studies on hemolytic anemias. They added 20 c. mm. of capillary blood to 1.0 c.c. of saline, or 0.1 c.c. of venous blood to 5.0 c.c. of saline; the resulting cell suspension was mixed with an equal volume of potent anti-A or anti-B serum, allowed to stand an hour, centrifuged, shaken, and again centrifuged. Anti-M and anti-N sera were used in the same way with the exception that the serum-cell mixtures were allowed to stand two hours and were not placed in the centrifuge.

The technique of Dacie and Mollison was used with notable success by Brown, Hayward and co-workers[13] and by Callender and associates[14] in their investigations of the manner of destruction of transfused normal erythrocytes by normal and anemic recipients. This method also has been employed by Young and Lawrence[15, 16] whose only deviation was that of preparing an initial 1:101 dilution of blood in saline instead of a 1:51 dilution as recommended by Dacie and Mollison.

Other techniques of differential agglutination have been developed and successfully used by Dekkers,[17] Denstedt and co-workers,[18] and Thalhimer.[19] Ebert and Emerson,[20] who made a thorough study of the hemolytic effect of Group O blood and pooled plasma, were the first to describe the use of dried antiserum for differential agglutination, an innovation having advantages which will be enumerated subsequently. It is the technique of these authors[20, 21] that has been followed to the greatest extent in developing the method currently adopted by this laboratory.

In 1924 Isaacs[22] criticized studies employing the technique of differential agglutination of cells. He stated that reticulocytes and nucleated red cells were nonagglutinable and were largely responsible for the error of the method. Subsequent observations in other laboratories and those reported in this paper show clearly, however, that reticulocytes are readily agglutinated by the usual antisera.

APPLICATIONS OF THE TECHNIQUE

The applications of the technique of differential agglutination are described in detail in the publications to which references have been made and are reviewed briefly elsewhere.[23] These applications may be enumerated as follows:

1. Determination of the life span of normal red cells by transfusion to normal recipients. By this method the erythrocyte has been found to survive for periods as long as 120 days or more.[14]

2. Study of pathologic destruction of red corpuscles in hemolytic disorders by "cross-determination of the lifetime of erythrocytes."[12, 16, 24, 25] Normal cells are transfused to patients with hemolytic anemia and cells from the latter are transfused to normal recipients in an effort to determine whether intra- or extracorpuscular abnormalities, or both, may be present.

3. Investigation of transfusion reactions in which incompatible donated cells are rapidly destroyed by the action of regular or irregular isoagglutinins of the recipient, or in which the recipient's cells are subjected to the hemolytic effect of transfused plasma or Group O blood.[9, 20, 26, 27]

4. Evaluation of the effects of storage, preservatives, and methods of transport of whole blood and red blood cells.[8, 10, 11, 18]

5. Observations on the selective action of the spleen on abnormal autologous cells as contrasted with normal donated cells.[28, 29]

THE TECHNIQUE

Five cubic centimeters of venous blood are withdrawn into a dry syringe with a minimum of stasis, and after removing the needle, the blood is expelled into a bottle containing 4 mg. of dried potassium oxalate and 6 mg. of dried ammonium oxalate.[30] After gentle rotation, 0.5 c.c. of the oxalated blood is transferred with a volumetric pipette to a 100 c.c. volumetric flask which is then filled to the mark with fresh normal saline. After thorough mixing, 0.2 c.c. portions of the 1:200 suspension are transferred with a serologic pipette

to each of four test tubes of approximately 7 mm. inside diameter and 6 cm. length. Tubes 1 and 2 contain only the saline suspension of cells, while tubes 3 and 4 contain 2 to 4 mg. of dried anti-M or anti-A serum.* This is the amount which can conveniently be held on the broad end of a toothpick, and no more accurate measurement than this appears to be necessary or practical. All the tubes are shaken gently and after about three minutes tubes 3 and 4 containing the serum-cell mixtures are centrifuged for one minute at 1,000 revolutions per minute. They are then shaken for fifteen seconds by rapid flicking against the table and after standing five minutes or longer at room temperature the mixtures are counted.

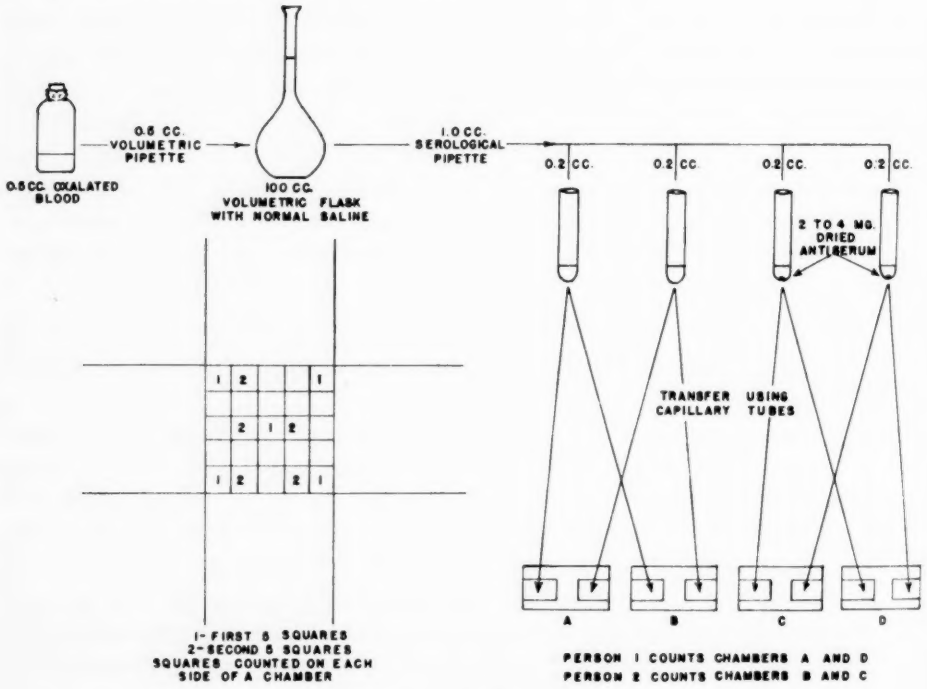

Fig. 1.—Diagram of technique.

After reshaking for fifteen seconds the cell suspensions and serum-cell mixtures are transferred to a bright-line counting chamber by means of large bore (0.8 to 1.2 mm.) capillary tubes which are discarded after a single filling. Each of two individuals fills one side of a chamber from each of the four tubes and counts the red cells in two sets of five squares (each square measuring 200 microns) in each side of a chamber as indicated in Fig. 1. By this procedure, 4 saline and 4 serum counts of five squares each are made by each person, thus giving a total of 8 saline and 8 serum counts. In counting fields where agglutinated cells are present, any square containing a large clump is omitted and an adjacent square is counted in its place. Both persons enumer-

*The dried antisera used in these studies were purchased from Lederle Laboratories, Pearl River, N. Y.

ate the cells simultaneously and with a minimum of delay. Counts are discarded and repeated if (1) either person suspects a break in technique or (2) the difference between the average counts for five squares is greater than 1.5 standard deviations.

The same lot of serum is used throughout each investigation, if possible, and the serum is tested at the beginning of the experiment, and periodically thereafter, for its ability to agglutinate the cells which possess the specific agglutinogen. If less than 10,000 per cubic millimeter of the cells containing the agglutinogen for which the antiserum is specific are left unagglutinated, no correction factor is necessary. If from 10,000 to 50,000 cells per cubic millimeter are left unagglutinated, this correction factor is subtracted from each serum count. If more than 50,000 cells per cubic millimeter fail to agglutinate, the serum is considered unsatisfactory for use in this procedure. This means that 99 per cent of the cells must be agglutinated if the suspension used contains 5 million cells per cubic millimeter.

The calculations made after completing each group of counts are most easily presented by citing an example such as that of an OMN recipient transfused with ON erythrocytes. Following such a transfusion, anti-M serum agglutinates nearly all of the recipient's cells and leaves unagglutinated all of the donated cells which are enumerated in the serum count. In this case:

(1) Total number of red cells per cubic centimeter (that is, OMN + ON cells) = (Average of 8 saline counts of five squares each) × 10,000
(2) Number of ON cells per cubic millimeter = (Average of 8 serum counts × 10,000) − the correction factor
(3) Number of OMN cells per cubic millimeter = Total number red cells per cubic millimeter − number ON cells per cubic millimeter

If typical figures are substituted in these formulas as examples, the following results are obtained:

(1) $425 \times 10{,}000 = 4{,}250{,}000$
(2) $(170 \times 10{,}000) - 20{,}000 = 1{,}680{,}000$
(3) $4{,}250{,}000 - 1{,}680{,}000 = 2{,}570{,}000$

It is to be noted that the average of the saline counts made by this method provides an accurate count of the total red cells per cubic millimeter when the figure is multiplied by 10,000.

If the number of unagglutinated cells in the serum-cell mixture is below 100 per set of five squares, it is advisable, because of large Poisson errors, to make a separate saline suspension containing 1, 2, or 4 c.c. of oxalated blood in 100 c.c. of normal saline and to use this suspension for mixing with antiserum. Counts obtained are then divided by 2, 4, or 8, respectively, in the final calculations.

When liquid antiserum is used instead of the dried preparation, 1.0 c.c. of oxalated blood is ordinarily diluted to 100 c.c. with saline and 0.2 c.c. of the 1:100 suspension is placed in each of four tubes as is done when dried serum is used. To tubes 1 and 2 is added 0.2 c.c. of saline and to tubes 3

and 4 is added 0.2 c.c. of antiserum. The remainder of the procedure is the same with liquid sera, with the exception that it may be necessary to shake the serum-cell mixtures more gently than when dried antisera are used. The optimum amount of shaking may vary from serum to serum, either wet or dry, and it is therefore necessary that this amount be determined for each lot of serum at the beginning of an experiment.

FACTORS AFFECTING THE ACCURACY OF THE TECHNIQUE

Selection of Donor-Recipient Combination Prior to Transfusion.—Since anti-A, -B, and -M sera give quantitative results, as will be shown in the discussion of antisera, it is possible to use differential agglutination in the combinations given in Table I. Inasmuch as the cells of all individuals in the population contain either the M or the N factor, or both, it is possible to carry out this procedure with all recipients. When Group O blood is given to A, B, or AB recipients, it is advisable to use only the red cells[20] or to add A and B substances to whole blood. The deliberate transfusion of Rh-positive blood into Rh-negative recipients for the purpose of studying differential agglutination is potentially dangerous and unjustifiable, particularly if there are to be repeated transfusions or if pregnancy is anticipated in the case of the Rh-negative female recipient. Likewise, the repeated transfusion of A_1 blood to an A_2 recipient or vice versa may be potentially dangerous, especially if the recipient is suffering from a hemolytic disorder.[31]

TABLE I. MIXTURES OF DONOR'S AND RECIPIENT'S BLOOD MOST SATISFACTORILY USED IN DIFFERENTIAL AGGLUTINATION

RECIPIENT'S GROUP	DONOR'S GROUP	SERUM USED TO DIFFERENTIATE	UNAGGLUTINATED CELLS
A	O	Anti-A	O
B	O	Anti-B	O
AB	O	Anti-A, Anti-B, or both	O
	A	Anti-B	A
	B	Anti-A	B
N	M	Anti-M	N
	MN	Anti-M	N
M	N	Anti-M	N
MN	N	Anti-M	N

Sampling of Recipient's Blood.—Best results have been obtained with 5 c.c. samples of venous blood drawn into dry, sterile syringes without stasis and transferred to bottles containing dry oxalate mixture. Capillary blood may be used if venous blood is not readily obtainable, but it is to be kept in mind that some accuracy is sacrificed in taking small samples.* Capillary blood is most easily drawn with a hemoglobinometer pipette from which a 0.02 c.c. sample is transferred to a tube containing 4.0 c.c. of saline, thus giving a

*It should be emphasized that the hematocrits of venous and capillary blood may differ significantly and that both may differ widely from the "whole body" hematocrit. The results of differential agglutination tests made after transfusion should be interpreted with these hematocrit variations in mind.

1:200 dilution of cells as in the method employing venous blood. Capillary samples are best taken and diluted in duplicate in order to reduce the error inherent in the use of small volumes.

Preparation of the Saline Suspension.—The 0.5 c.c. volumetric pipettes, which are used routinely in transferring venous blood samples to the 100 c.c. flasks, have an error of not more than 0.1 per cent, while ±.16 c.c. is the tolerance for error of the 100 c.c. volumetric flasks. Multiple dilutions with the accompanying increase in error are avoided by this method. It is important, if accurate results are to be obtained, that saline suspensions be of such density as to provide counts of between 400 and 500 and never less than 200 cells per five squares. Stronger suspensions should be made if the counts do not fall within this range. As previously stated, more concentrated suspensions of cells in saline should be prepared to mix with antiserum if the counts of unagglutinable cells fall below 100 per five squares. The coefficient of variation for a count of 100 is 9 per cent and is larger for counts smaller than 100.

Differential counts are sometimes made on the nonhemolyzed cells remaining in the bottom of each tube used in testing the osmotic or mechanical fragility of mixtures of two types of cells present in peripheral or splenic blood.[28, 29] When this is done, the cells are resuspended in normal saline until the density of the suspension matches that of a standard tube of the same size containing a 1:200 dilution of whole blood having a red cell count of from 4.0 to 5.0 million. Two-tenths cubic centimeter portions of the resuspended cells are then transferred to four small tubes, and the remainder of the procedure is carried out in the usual way. The proportions of the two types of cells can be determined from counts made on such preparations.

The Antiserum.—

The Antisera to Be Used: In light of the present knowledge of the agglutinogens of human erythrocytes, it is theoretically possible to use eleven different antisera for this purpose. These antisera are anti-A, -B, -A_1 (absorbed B), -M, -N, -P, -Rh_0, -Rh′, anti-Rh″, -Hr′, and -Hr″. In the final analysis the accuracy of the procedure depends to a great extent on the quality of antiserum used. Of the sera available in our laboratory only anti-M, -A, and -B have been satisfactory.

Dried serum has the advantages of relatively uniform potency, stability, and economy. The fact that dried antiserum, in the amounts used, does not dilute the saline cell suspensions was proven by comparing the counts of saline suspensions of AN cells before and after adding dried anti-M serum in the usual proportions. The difference between the counts was only 2.8 per cent, revealing no apparent dilution or nonspecific agglutination. This experiment was repeated using dried anti-N serum and AM cells and the difference in counts was less than 4 per cent.

The Proportion of Antiserum and Cell Suspension: The amount of dried serum, which causes nearly complete specific agglutination and no nonspecific agglutination, has been determined by our experiments to be from 2 to 4 mg. per 0.2 c.c. of cell suspension. Amounts of from 4 to 8 mg. in 0.1 c.c. of cell suspension caused nonspecific agglutination in the case of anti-N serum. For

practical purposes the amount which can be held on the broad end of a toothpick (2 to 4 mg.) gives satisfactory results.

Correction Factor for Nonagglutinable Cells: The problem of correction factors based upon counts of unagglutinated cells made prior to transfusion has been extensively dealt with in the literature.[5, 10, 22] To be satisfactory for use in this procedure the serum should leave unagglutinated not more than 50,000 cells per cubic millimeter, or more than 1 per cent of cells containing the specific agglutinogen. Frequent retesting of the antisera, particularly liquid antisera, to check the correction factor is advised. As shown in Table II, the nonagglutinable cells amounted to only 0.2 per cent with dried anti-M serum and 0.5 per cent with dried anti-A serum currently in use in this laboratory.

The theory that nonagglutinable cells are reticulocytes or nucleated cells is untenable in view of the work of Callender and associates[14] and the following observations made in this laboratory:

1. The freshly drawn erythrocytes which were left unagglutinated after mixture with anti-A, -B, and -M sera were stained with brilliant cresyl blue and no reticulocytes or morphologically abnormal cells were found.

2. Dried anti-M serum was added to a suspension of MN cells from a patient with chronic hemolytic anemia. Although 51 per cent of the cells were reticulocytes, only 2 per cent were left unagglutinated, which means that most of the reticulocytes were agglutinated as were the mature cells. At the beginning of a previous experiment,[15] when 73 per cent of the erythrocytes from this patient contained reticulum, only 0.5 per cent were left unagglutinated by liquid anti-M serum.

Potency of Antiserum: In order to test the effectiveness of various antisera in differential agglutination, suspensions of washed, fresh A_1MRh+, BNRh+, OMRh–, and A_2MRh+ cells were prepared in saline. Counts were made on each suspension, 1.0 c.c. portions of each suspension were combined to make a mixture of the four types of cells, and this mixture was then counted. To portions of the mixed cell suspension, anti-A, -B, -A_1, -M, -N, and -Rh_0 sera were added by the technique described. In order to determine the nonagglutinable cell count, each of these antisera was tested with that suspension of cells containing the agglutinogen for which it was specific; for example, the anti-B serum was tested with the BNRh+ cells. Four counts of five squares each were made by each of four individuals on each saline suspension and serum-cell mixture.

This experiment was designed to test the accuracy of differential agglutination with the antisera which were available in our laboratory on a given date. Although the results of this experiment which are shown in Table II apply only to the sera tested, similar observations may be carried out to compare the potency of other sera and to determine the accuracy of the procedure. It is apparent that only the anti-M and anti-A sera tested were sufficiently potent to agglutinate 99 per cent of the cells containing the specific agglutinogen. A comparison of expected per cent nonagglutinated cells and the actual per cent of nonagglutinated cells in the mixture of cells is shown in Table II. The

TABLE II. RESULTS OF DIFFERENTIAL AGGLUTINATION OF ARTIFICIAL MIXTURE OF ERYTHROCYTES WITH ANTISERUM

ANTISERA	AVERAGE OF 4 COUNTS BY EACH OF 4 PERSONS	ACTUAL PER CENT NOT AGGLUTINATED*	EXPECTED PER CENT NOT AGGLUTINATED	PER CENT DIFFERENCE + OR −	PER CENT NONAGGLUTINABLE†
Anti-A (dry)	258	52.6	50.4	2.2‡	0.5
Anti-B (dry)	372	71.4	73.8	2.4§	4.9
Absorbed B (liquid)	206 × 2	41.1	75.6	34.5§	41.7
Anti-M (dry)	122	24.9	26.2	1.3§	0.2
Anti-N (dry)	304	54.1	73.8	19.7§	8.21
Anti-Rh_0 (liquid)	125 × 2	26.6	24.2	1.8‡	26.2

*Corrected for nonagglutinable cells as shown in last column.
†Counts made on individual cell suspensions.
‡Not agglutinated.
§Agglutinated.

anti-A_1 and anti-N sera yielded results grossly different from those expected. These results illustrate the consequence of using antisera of low potency and specificity. The dried anti-B serum used in this experiment left 4.71 per cent of B cells unagglutinated, whereas previous lots of liquid anti-B serum obtained from donors immunized with A and B substances were considerably more potent. Additional lots of dried anti-B serum have not yet been tested in this laboratory.

Minimum requirements for blood grouping and typing sera have recently been set forth by the National Institute of Health.[32]

The Reaction of Specific Agglutinin and Agglutinogen.—

Time: The time required for maximal agglutination varies widely depending upon the avidity of the serum and whether or not the serum-cell mixture is centrifuged. In our experience and that of other recent investigators,[21] three minutes before centrifuging and five minutes afterward are adequate.

Centrifugation of Serum-Cell Mixtures: It is well known that hemagglutination is greatly accelerated by centrifugation of serum-cell mixtures. Dacie and Mollison[12] recommend that the mixtures containing liquid anti-A or anti-B serum be allowed to stand for at least sixty minutes before centrifugation. The experience of this laboratory indicates, however, that a period as short as three minutes is adequate for dried anti-A and anti-M sera and probably for liquid anti-A, anti-B, and anti-M sera. Ebert and Emerson[20] and Emerson and Shen[21] have also found that it is necessary for mixtures containing dried anti-A or anti-B serum to stand only a few minutes before centrifugation.

Since anti-M and anti-N sera are absorbed rabbit sera, nonspecific reactions between these sera and human cells may often be encountered.[3, 19] Dacie and Mollison have cautioned against centrifugation of mixtures containing anti-M or anti-N serum because of the danger of causing nonspecific clumping of type N cells by an occasional lot of liquid anti-M serum, but this has not been true of the lots of dried anti-M serum thus far tested. Both liquid and dried anti-N sera have been noted to cause some nonspecific agglutination in our experience, particularly after centrifugation. If centrifugation is carried out for more than one minute at 1,000 to 1,500 r.p.m. there may be some false agglutination.

Temperature: In our experience and that of other workers[2, 10] room temperature is most satisfactory. The titer, and probably the avidity of isoagglutinins is decreased at temperatures as high as 44 to 55° C. which were recommended by Jervell.[6] Refrigeration, on the other hand, as originally used by Ashby[1] may cause nonspecific cold hemagglutination.

Shaking: The amount of shaking to which the serum-cell mixture should be subjected is a problem which must be standardized with various sera. We have found that with potent dried antisera vigorous shaking by flicking against the table for fifteen seconds is necessary and if the shaking is too gentle false agglutination may be present. Vigorous shaking may not be necessary, however, when liquid antisera are used. Saline suspensions are shaken in the same fashion as serum-cell mixtures.

Filling the Chamber.—For filling the chamber it has been found that disposable glass capillary tubes which are from 0.8 to 1.2 mm. in diameter and 12 cm. in length are convenient and give constant results. When exceptionally large clumps of agglutinated cells are present, it is advisable to avoid them in removing samples from the tubes.[2, 12] When saline cell suspensions were transferred to counting chambers with 1.2 mm. capillary tubes, 0.2 mm. capillary tubes and the stems (0.4 mm.) of Trenner erythrocyte pipettes, the differences in counts were insignificant. In making this comparison each of two persons made four transfers by each of the three methods and counted ten (200 mm.) squares after each transfer. It was found, however, that in transferring serum-cell mixtures irregularities were apt to occur when capillary tubes smaller than 0.5 mm. in diameter were used. Trenner pipettes have not been employed for this purpose because of the time required for their cleaning.

Counting of Cells.—Simultaneous counts by two persons of the cells in each tube make it possible to detect the unavoidable errors of technique which occasionally occur. These errors are assumed to exist, even though they have not been observed, when the differences between the counts is greater than 1.5 standard deviations. The counts are then discarded and repeated with different chambers. The 1.5 standard deviation control limit is economical, permitting a loss of only about 12 per cent of "true" counts. The standard deviation of the difference between the counts (that is, the average of four sets of five squares) obtained by two persons is:

$$\sigma\ (A_1 - A_2) = \sqrt{.015\ (A_1^2 + A_2^2) + .2\ (A_1 + A_2)}$$

where A_1 and A_2 are the counts by the two persons (See Statistical Considerations). In the range of counts dealt with, 100 to 500, 1.5 σ has the range 20 to 80.

In order to determine the better method a statistical test was done comparing: (1) two persons counting ten squares on each side of the chamber and (2) two persons counting twenty-five squares on only one side of the chamber. By each method 30 saline suspensions of different concentrations were counted. The coefficients of variation for the difference between the average number of cells in five squares (that is, the average counts) found by two persons were corrected for Poisson errors and were found to be (1) 8.2 per cent and (2)

13.4 per cent. Fisher-Z which tests the homogeneity of these values has a probability near 5 per cent. The discrepancy of these values is attributable to chamber error, more thoroughly characterized by Berkson[33] as 4.5 per cent. Since the consistency between counts by different persons is used as a criterion of statistical control, the first method (1) was adopted because of its greater precision.

STATISTICAL CONSIDERATIONS

Formulation of the technique presented in this paper was based upon many practical and experimental considerations, some of which required statistical tests and judgments in the management of sampling errors in counts. A count obtained by this technique presumably will be compared with another one under different experimental or clinical conditions. Such comparisons of counts require statistical judgments based on the standard deviations of counts and differences between them. If σ_A is the standard deviation of count A, and σ_B is the standard deviation of count B, the standard deviation of the difference between counts A and B is:

$$\sigma_{(A-B)} = \sqrt{\sigma_A^2 + \sigma_B^2}$$

Similar manipulations of the standard deviation can be found in elementary statistical textbooks for other types of experimental comparisons. A 1.5 σ tolerance or error range includes about 88 per cent of random counts. In this laboratory differences between counts beyond the 1.5 σ range are conventionally considered nonrandom.

The general formula for the standard deviation of a count by the technique of this paper is given by:

$$\sigma_A = \sqrt{\frac{0.15\ A^2}{N} + \frac{.8A}{MN}}$$

where A is the count, which is the average count of M counts of five squares by each of N persons. The precision of any statistical test using σ_A can be enhanced by increasing M, N, and/or A. A convenient number of counts of five squares is 4(M = 4) and persons is 2(N = 2), and counts are held within the range 100 to 500. The first factor (.015) was determined from several hundred counts made under varying circumstances: different values of M, N, and A; saline and different antisera; different recipient-donor combinations, etc.; all of these conditions showed homogeneity of errors. This factor, which should be determined in each laboratory unless the technique described here is followed exactly, represents the relative variation due to random effects in apparatus and technique and limits the precision in high counts. The second error factor (.8A) due to Poisson errors[33] is important in low counts. For A = 100, σ_A = 9. Thus, counts smaller than 100 should be avoided by using cell suspensions of sufficiently low dilution.

Example: Accompanying the numerical example under Technique are the following calculations of errors:

(1) Saline count $= 425 \pm 37$

where $37 = \sigma = \sqrt{\frac{0.15\ (425)^2}{2} + \frac{(.8)\ (425)}{(4)\ (2)}}$

(2) Serum count $= 168 \pm 15$

where $15 = \sigma = \sqrt{\frac{.015\ (168)^2}{2} + \frac{(.8)\ (168)}{(4)\ (2)}}$

(3) Saline minus Serum $= 257 \pm 40$

where $40 = \sigma = \sqrt{(37.)^2 + (15)^2}$

In similar fashion the difference between (1), (2), or (3) counts for different clinical or experimental conditions can be tested for statistical significance by repeated application of the general formula, $\sigma_{(A-B)}$, previously given.

Example: One day after transfusion of ON blood to an OMN recipient, the technique was carried out using dried anti-M serum which agglutinated the recipient's but not the donor's cells. The serum count was 132 and a month later it was 103. The standard deviation for the difference between two counts, A = 132, B = 103:

$$\sigma_{(A-B)} = \sqrt{\frac{.015\ (132^2 + 103^2)}{2} + \frac{.8\ (132 + 103)}{(4)\ (2)}} \cong 15$$

SUMMARY

1. A technique of differential agglutination of human erythrocytes is presented in detail and its applications in experimental medicine are outlined.

2. The accuracy of the technique has been tested experimentally.

3. The precision of the technique has been analyzed and stated statistically.

CONCLUSIONS

1. The technique of differential agglutination gives accurate and precise quantitative results.

2. Antisera of sufficient potency, avidity, and specificity are necessary. Dried antisera (A, B, and M) have the advantages of stability and relatively uniform potency.

3. Reticulocytes are agglutinated in the same manner as mature cells and erythrocytes which fail to agglutinate are not morphologically abnormal.

REFERENCES

1. Ashby, W.: The Determination of the Length of Life of Transfused Blood Corpuscles in Man, J. Exper. Med. **29**: 267, 1919.
2. Wiener, A. S.: Correspondence, J. A. M. A. **122**: 1779, 1934.
3. Wiener, A. S.: Blood Groups and Transfusion, ed. 3, Baltimore, Md., and Springfield, Ill., 1943, Charles C Thomas.
4. Ashby, W.: Some Data on the Range of Life of Transfused Blood Corpuscles in Persons Without Idiopathic Blood Diseases, M. Clin. North America **3**: 783, 1919.
5. Wearn, J. T., Warren, S., and Ames, O.: The Length of Life of Transfused Erythrocytes in Patients With Primary and Secondary Anemia, Arch. Int. Med. **29**: 527, 1922.
6. Jervell, F.: Untersuchungen über die Lebensdauer der Transfundierten Roten Blutkörperchen beim Menschen, Acta path. et microbiol. Scandinav. **1**: 155, 1924.
7. Landsteiner, K., Levine, P., and Janes, M. L.: On the Development of Isoagglutinins Following Transfusions, Proc. Soc. Exper. Biol. & Med. **25**: 672, 1928.

8. Wiener, A. S., and Schaefer, G.: Limitations in the Use of Preserved Blood for Transfusion, M. Clin. North America **24**: 705, 1940.
9. Wiener, A. S., and Peters, H. R.: Hemolytic Reactions Following Transfusions of Blood of the Homologous Group With 3 Cases in Which the Same Agglutinogen Was Responsible, Ann. Int. Med. **13**: 2306, 1940.
10. Mollison, P. L., and Young, I. M.: On the Survival of the Transfused Erythrocytes of Stored Blood, Quart. J. Exper. Physiol. **30**: 313, 1941.
11. Mollison, P. L., and Young, I. M.: In Vivo Survival in the Human Subject of Transfused Erythrocytes After Storage in Various Preservative Solutions, Quart. J. Exper. Physiol. **31**: 359, 1942.
12. Dacie, J. V., and Mollison, P. L.: Survival of Normal Erythrocytes After Transfusion to Patients With Familial Haemolytic Anaemia (Acholuric Jaundice), Lancet **1**: 550, 1943.
13. Brown, G. M., Hayward, O. C., Powell, E. O., and Witts, L. J.: The Destruction of Transfused Erythrocytes in Anemia, J. Path. & Bact. **56**: 81, 1944.
14. Callender, S. T., Powell, E. O., and Witts, L. J.: The Life Span of the Red Cell in Man, J. Path. & Bact. **57**: 129, 1945.
15. Young, L. E., and Lawrence, J. S.: Maturation and Destruction of Transfused Human Reticulocytes; Evaluation of Reticulocyte Experiments for the Measurement of Hemoglobin Metabolism, J. Clin. Investigation **24**: 554, 1945.
16. Young, L. E., and Lawrence, J. S.: Atypical Hemolytic Anemia; Observations With Particular Reference to the Use of Transfusions in the Study of Hemolytic Mechanisms, Arch. Int. Med. **77**: 151, 1946.
17. Dekkers, H. J. N.: The Fate of the Transfused Red Blood Cells, Acta med. Scandinav. **99**: 587, 1939.
18. Denstedt, O. F., Osborne, D. E., Rochlin, I., and Stansfield, H.: The Survival of Preserved Erythrocytes After Transfusion, Canad. M. A. J. **48**: 477, 1943.
19. Thalhimer, W.: Personal communication.
20. Ebert, R. V., and Emerson, C. P., Jr.: A Clinical Study of Transfusion Reactions: the Hemolytic Effect of Group O Blood and Pooled Plasma Containing Incompatible Isoagglutinins, J. Clin. Investigation **25**: 627, 1946.
21. Emerson, C. P., and Shen, S. C.: Personal Communications.
22. Isaacs, R.: Properties of Young Erythrocytes in Relation to Agglutination and Their Behavior in Hemorrhage and Transfusion, Arch. Int. Med. **33**: 193, 1924.
23. Young, L. E.: The Red Blood Cell as an Immunologic Tool, Indust. Med. **15**: 590, 1946 (abstract). Manuscript in preparation.
24. Singer, K.: Problems of Erythrocyte Disintegration With Particular Reference to the Life Span of the Red Cell, J. Lab. & Clin. Med. **30**: 784, 1945.
25. Loutit, J. F.: Discussion on the Life and Death of the Red Blood Corpuscle, Proc. Roy. Soc. Med. **39**: 757, 1946.
26. Mollison, P. L.: The Investigation of Hemolytic Transfusion Reactions, Brit. M. J. **1**: 529, 1943.
27. Young, L. E., and Kariher, D. H.: Hemolytic Transfusion Reactions Due to Rh Incompatibility, J. A. M. A. **127**: 627, 1945.
28. Emerson, C. P., Jr., Shen, S. C., and Castle, W. B.: The Osmotic Fragility of the Red Cells of the Peripheral and Splenic Blood in Patients With Congenital Hemolytic Jaundice Transfused With Normal Red Cells, J. Clin. Investigation **25**: 922, 1946.
29. Young, L. E., Platzer, R. F., and Lawrence, J. S.: Unpublished Observations.
30. Heller, V. E., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants, J. Lab. & Clin. Med. **19**: 777, 1934.
31. Young, L. E.: Studies of the Subgroups of Blood Groups A and AB. I. Active and Passive Acquisition of Alpha, Agglutinins as a Result of Transfusion, J. Immunol. **51**: 101, 1945.
32. National Institute of Health: Minimum Requirements for Blood Grouping Serum and Anti-Rh Typing Serum, Dec. 16, 1946.
33. Berkson, J.: Medical Physics, Chicago, 1944, The Year Book Publishers, Inc., p. 110.

FIBRINOGEN DEFICIENCY: CLINICAL FEATURES AND PROBABLE ETIOLOGIC FACTORS

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IN EVALUATING factors which are responsible for a hemorrhagic state, fibrinogen deficiency is seldom considered. Yet, occasionally, as in the case to be described later, it may be an important factor.

Fibrinogen deficiency is rare. It may be either acquired or congenital. Quick[1] described the condition and reviewed the literature. The congenital type is characterized by its hereditary nature, by the tendency to bleed intermittently from birth, frequently oozing from the umbilical stump, and by a low or absent fibrinogen. Nine cases[2] have been reported with no fibrinogen and five[3] with a reduced quantity.

The acquired form has been reported more frequently. Such instances usually show a transient fibrinogen deficiency that has been attributed to an interference in fibrinogen formation. In 1924 Opitz and Silberberg[4] described a fatal case of generalized tuberculosis with hemorrhagic diathesis in a 3-year-old girl with no fibrinogen. At autopsy the liver was replaced by caseous masses and this finding was considered by the authors as evidence that the liver is the source of fibrinogen. In 1927 Knauer[5] reported a case of possible purpura fulminans occurring in a 6-year-old girl during an attack of varicella. Fibrinogen was 0.015 Gm. per 100 c.c. of plasma. No liver damage was evident and it was suggested that the fibrinogenopenia was due to reticuloendothelial system damage. In 1930 Jürgens and Trautwein[6] found less than 0.1 Gm. per cent fibrinogen in a 52-year-old man with extensive prostatic metastases in the bone marrow. The liver showed subcapsular deposits but otherwise was normal. They suggested that bone marrow has a role in the manufacture of fibrinogen. In 1935 Risak[7, a] examined 230 patients with varied diagnoses and found eighteen with a fibrinogen level below 0.1 Gm. per cent; most of these did not show a bleeding tendency. They included patients with blood dyscrasias, infectious diseases, extensive burns, malignancies, and a miscellaneous group. Waldenstrom[7, b] has recently described three patients with fibrinopenia, hyperglobulinemia, and a tendency to bleed excessively.

In the English literature in 1936 Dieckmann[8] reported three cases of abruptio placentae with a fibrinogen level below 0.1 Gm. per cent and attributed this to the loss of blood. Allibone and Baar[3, b] have described a complete absence of fibrinogen in an infant with obliteration of the bile ducts and biliary cirrhosis of the liver. The fibrinogen was restored to normal with treatment; however, progressive liver damage continued and death occurred in three months. They reviewed the literature, found fibrinogen normal in cases of severe liver damage, and concluded that a decrease in fibrinogen may or may not occur when the liver is diseased and that the site of formation in the liver

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and bone marrow is fairly well established. Recently in 1946 Field and Dam[9] reviewed the factors in production of fibrinogen and concluded that in the chick the fibrinogen levels are influenced by dietary regimen. Apparently an inadequate protein intake produced elevated fibrinogen levels which were attributed to a metabolic disturbance of the liver.

The fibrinogen level at which a hemorrhagic state appears is controversial. In 1931 Peters and Van Slyke[10] gave the normal range for plasma fibrinogen as 0.2 to 0.4 Gm. per cent. In 1930 Jürgens and Trautwein[6] considered 0.15 to 0.12 Gm. per cent as the critical level below which blood did not coagulate. Risak[7, a] cited a patient with constitutional fibrinogenopenia whose son had only 0.02 Gm. per cent fibrinogen and no bleeding tendencies. In many cases the bleeding is intermittent even though the fibrinogen remains low or even absent. This inconsistency has been attributed to the existence of additional factors. Rabe and Salomon[2, a] thought some change in vascular permeability may be necessary. Several authors[2, b, d, e, f, h] have commented on the association of a thrombopenia and decreased fibrinogen. Allibone and Baar[3, b] reported a case of congenital fibrinogenopenia with hemorrhagic manifestations with a value of 0.11 Gm. per cent and reviewed the theory that a deficiency in the factor of the coagulation mechanism may be compensated by the remaining factors. Henderson and associates[2, h] concluded that the threshold is about 0.06 Gm. per cent.

Because the number of cases so far reported is small, a recent case encountered by us is described in detail.

CASE HISTORY

O. W.,* a 51-year-old farmer's wife, was first admitted to Wesley Memorial Hospital on Aug. 8, 1944, with complaints of diarrhea and generalized weakness both of several months duration. No evidence of a hemorrhagic tendency was present at that time, and the diagnosis was multiple dietary deficiency resembling sprue. The patient was discharged on a high caloric, high protein, high vitamin diet plus supplemental vitamins. On this therapy the diarrhea subsided in about a month and she felt greatly improved, so much so that in May, 1945, nine months after the first admission, she stopped the specific diet and the vitamin supplements. However, the food intake remained abundant and varied. Late in June, 1945, the diarrhea recurred and the weight dropped from 116 to 100 pounds. In August, ten days prior to the second entry, large black and blue areas appeared on the skin. A few days later the patients knees became painful and discolored. Two small skin abrasions oozed constantly. On the evening before admission she injured her gum with a pin, producing bleeding which continued until about thirty-six hours after entry.

At the time of admission the patient was emaciated. Ecchymotic areas covered all extremities and were numerous about the head. Abrasions on the arm were oozing slightly. The gum at the site of the injury was bleeding freely. The knees were swollen and tender. The skin of the hands, legs, and feet was unusually firm and thick. The blood pressure was 118/80. No abnormalities of the heart, lungs, and abdomen were noted on physical examination. Throughout the next two days bleeding into the tissues, from the gum and urinary tract, became so severe that termination of the hemorrhagic state was urgently needed.

Factors involved in the coagulation mechanism† were considered. As noted in Table I, the coagulation time was seventy minutes. Since calcium is almost invariably present in sufficient concentration to promote thrombin formation when the other factors are present, a possible calcium deficiency seemed unlikely as the cause. A low ascorbic acid level could

*Patient seen through the courtesy of Dr. Edgar C. Cook of Mendota, Ill.

†Prothrombin plus calcium plus thromboplastin equals thrombin; fibrinogen plus thrombin equals fibrin.

have accounted for increased capillary fragility but not for a prolonged coagulation time. The platelets were only slightly reduced in number (although they may have been functionally abnormal). Consequently, at first the defect was thought to be a decreased prothrombin concentration. Ten milligrams of vitamin K were administered intravenously at eight-hour intervals. A blood transfusion was given three hours after the first dose of vitamin K. The transfusion was followed by immediate cessation of bleeding. It is impossible to state the degree to which the vitamin K, on one hand, and the blood transfusion, on the other, contributed to the termination of bleeding.

This patient has returned for observation on two occasions. There has been no further bleeding, the weight remained unchanged, but she still has irregular periods of diarrhea. Firmness and thickness of the skin of the hands and forearms have persisted.

The significant laboratory findings are tabulated in Tables I to IV. A fibrinogen level of 0.04 Gm. per cent was reported; this value is well below the critical level indicated by most authors. Consequently, it seemed probable that fibrinogen deficiency was an important factor in the bleeding tendency of this patient. During the acute bleeding episode hematuria was noted. Small amounts of free hydrochloric acid were present in the gastric contents on each admission. Stool analysis and proctoscopy showed no abnormalities. The total fat content of dried feces was 22 per cent. The bone marrow showed no significant change; erythropoiesis was normoblastic.

A deficiency in both fibrinogen and prothrombin probably existed simultaneously in this patient. Estimation of either substance depends on the presence of the other essentials of the clotting mechanism. Spontaneous clot formation did occur in the calcified plasma used for fibrinogen determination, and sufficient repetition of analyses by two different methods in separate laboratories verified the exceedingly low level of fibrinogen in the blood. A comparison of the results obtained by the addition of thrombin and by allowing spontaneous coagulation to occur after recalcification of the plasma indicate that a slight difference is obtained by the two methods on either fibrinopenic or normal blood. Spontaneous coagulation was delayed for hours in the most fibrinopenic blood, and the clot formed at the lowest fibrinogen levels reported did not possess the physical properties ordinarily observed in freshly coagulated fibrin. In our experience the most satisfactory method of fibrin estimation is the direct determination of nitrogen in the coagulum after it has been collected and washed. The addition of calcium to plasma results in a somewhat higher value for fibrin as compared to that obtained when thrombin alone is added. This difference, although representing only 0.02 to 0.04 Gm. per cent fibrin, was quite consistently obtained on different samples of blood. Since the determination of prothrombin assumes a normal fibrinogen concentration, prothrombin estimation here would be in error. In only a few of the previous cases of fibrinopenia reported by other authors has prothrombin been considered. Allibone and Baar[3, b] reported no clot in their prothrombin estimations and discussed the relationship between prothrombin and other coagulation factors. They also pointed out that the routine

TABLE I. COAGULATION FACTORS

	8/8/44	8/18/45	8/22/45	8/24/45	8/24/45	8/27/45	11/9/45	2/11/46
Fibrinogen[13] (grams per cent)		0.04*	0.06	0.078*	0.18	0.18	0.24	0.20
Coagulation time in minutes (Lee-White)		70	6	10			5	5
Prothrombin time in seconds[14, 15]								
Patient		No clot	22	70			30	32
Normal		25	23	35			27	26
Bleeding time in minutes (Duke)		3½						
Clot retraction (twenty-four hours)		Slight						Normal
Platelet count (cubic millimeters)		220,000	140,000			146,000	205,000	161,000
Rumpel-Leede				Positive				
Ascorbic acid	0.16	0.26				1.32	0.88	1.22

*Blood transfusions were given August 19 and 24.

estimation of prothrombin assumes a normal fibrinogen. Quick[1] has stated that the bleeding in acute yellow atrophy frequently attributed to low fibrinogen is very likely due to severe hypoprothrombinemia.

Further studies were made in an effort to determine the cause of the fibrinogen and prothrombin deficiencies. Liver function was only slightly impaired as judged by the tests employed (Table II). The changes in liver function which were observed are such as one might expect to find in chronic impairment of intestinal absorption. The chronic diarrhea, gross evidence of malnutrition, flat oral glucose tolerance curve (as compared to the normal intravenous curve, Fig. 1), and low plasma vitamin A and carotene levels are all evidence of impaired gastrointestinal absorption. The moderate macrocytic anemia (Table III) and x-ray findings of dilated loops of intestine (Fig. 2) are other manifestations compatible with the diagnosis of a nutritional deficiency which would necessarily be due to impaired absorption since the patient had been on an adequate diet.

The change in intestinal absorption may have been due to a "generalized scleroderma"[11] involving the intestine as well as the skin. However, a skin biopsy showed no abnormality other than edema. The patient's physician recently reported that the skin of the lower legs and feet has become markedly thicker during the months after she left the hospital.

Sprue was considered as a diagnosis. However, at no time did the patient complain of glossitis, and free acid was always found in the gastric contents. Nevertheless, the low plasma vitamin A and carotene levels and the presence of macrocytosis suggest sprue.

Intestinal absorption may have been affected by the pancreatic insufficiency as indicated by the poor amylase output to intravenous secretin (Table IV). The lack of therapeutic response to oral pancreatin, however, argues against pancreatic function as other than a contributory factor to the picture. The findings of fibrinogenopenia in the absence of marked

TABLE II. STUDIES OF HEPATIC FUNCTION

	8/8/44	8/20/45	8/27/45	11/7/45	2/11/46
Bromsulphalein (thirty minutes)			Negative		
I.V. hippuric acid test (0.7 to 0.95 Gm.)			0.3595	0.3469	0.4539
Cephalin flocculation					Negative
Total plasma protein (grams per cent)	4.68	6.18	5.72	5.30	6.03
Albumin	1.2		3.04	3.3	3.81
Globulin	3.48		2.68	2.0	2.04
Icterus index				6.5	

TABLE III. COMPOSITION OF BLOOD

	8/8/44	8/17/45	8/21/45	8/27/45	11/6/45	2/11/46
R.B.C. (million per cubic millimeter)	3.15	4.0	3.92	4.29	3.67	3.59
Hemoglobin (grams)	10	13.0		13.0	12.5	12.0
W.B.C. per cubic millimeter	3,450	6,050		6,250	5,000	4,750
Macrocytosis		Present			Present	Present
Hematocrit per cent					45	41
Urea nitrogen (milligrams per cent)	14.1			10.3		
Serum calcium					10.5	8.1
Pyruvic acid (0.55 to 0.95 mg.)		0.8				0.8
Carotene (100 to 350 I.U.)		13				0
Vitamin A (100 to 250 I.U.)		47				60

TABLE IV. PANCREATIC AMYLASE IN PATIENT AND NORMAL ADULT

SAMPLE	DUODENAL FLUID		AMYLASE		NORMAL RESPONSE
	TOTAL	C.C./MIN.	CONCENTRATION	OUTPUT	OUTPUT
Before secretin*	10	0.5	0	0	30,000
Ten minutes after	28	2.8	600	16,800	77,000
Twenty minutes after	5	0.5	690	3,470	16,300
Thirty minutes after	6	0.6	840	5,050	17,400

*Response to intravenous secretin expressed as milligrams of maltose liberated per cubic centimeter of pancreatic juice.[13] We are indebted to Dr. H. G. Greengard for the secretin used in this test and for the amylase determinations.

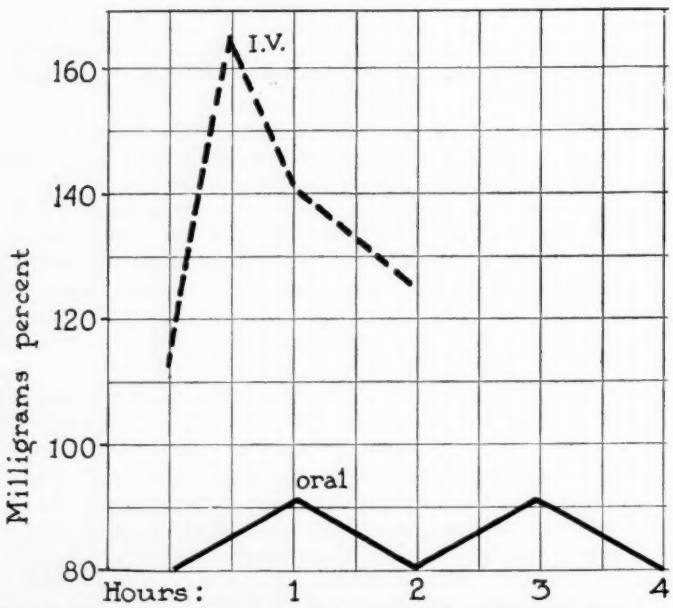

Fig. 1.—Comparison of oral and intravenous glucose tolerance curves.

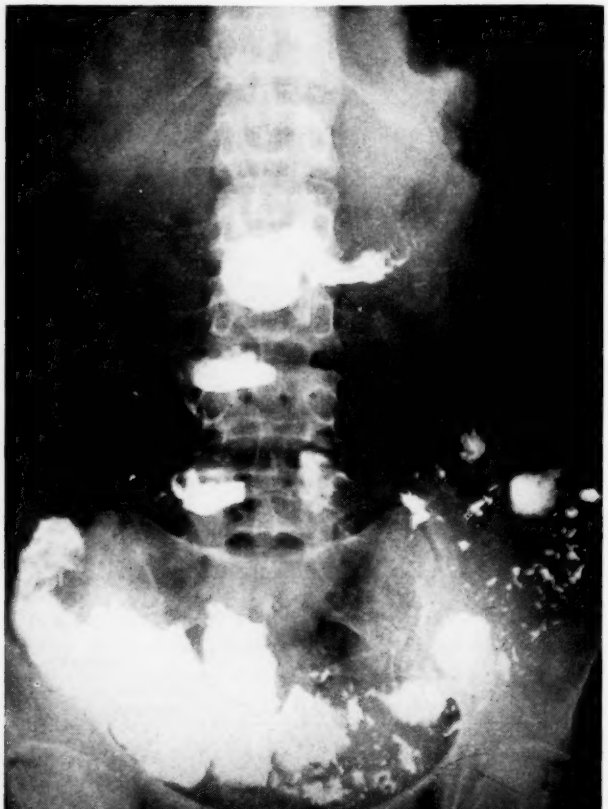

Fig. 2.—Persistence of barium in the small bowel and duodenum five hours after ingestion.

hepatic insufficiency coupled with impaired intestinal absorption suggests that there may be some nutritional factor that is essential for the formation of fibrinogen by the body.

Treatment has consisted of supplemental and replacement measures. No further bleeding has been reported.

SUMMARY

A review of the literature reveals a paucity of reports in which fibrinogenopenia was proved to be an important factor in the production of a severe hemorrhagic state. It is for this reason that this instance is reported in detail. Whether the low fibrinogen level was due to the inability of the liver to synthesize this protein or to impaired absorption of its precursors from the gastrointestinal tract is not known. The evidence points to the latter cause. The cause of poor absorption in this case may be sclerosis of the intestinal wall similar to the changes recognized as scleroderma in the skin.

REFERENCES

1. Quick, A. J.: The Hemorrhagic Diseases and the Physiology of Hemostasis, Springfield, Ill., 1942, Charles C Thomas, Publisher, pp. 184-191.
2. (a) Rabe, F., and Salomon, E.: Ueber Fasterstoff mangel im Blute bei einem Falle von Hämophilie, Deutsches Arch. f. klin. Med. **132**: 240, 1920.
   (b) Opitz, H., and Frei, M.: Ueber eine neue Form der Pseudohämophilie, Jahrb. f. Kinderh. **94**: 374, 1921.
   (c) Wolff, J.: Verblutungstod wegen Fibrinmangels, Jahrb. f. Kinderh. **148**: 33, 1936.
   (d) Macfarlane, R. G.: A Boy With No Fibrinogen, Lancet **1**: 309, 1938.
   (e) Schönholzer, G.: Die hereditäre Fibrinogenopenie, Deutsches Arch. f. klin. Med. **184**: 496, 1939.
   (f) Glanzmann, E., Steiner, H., and Keller, H.: Konstitutionelle angeborene Afibrinogenämie und Fibrinopenie im Kindesalter, Schweiz. Med. Wchnschr. **70**: 1243, 1261, 1940.
   (g) Castex, M. R., Pavlovsky, A., and Bonduel, A.: Fibrinopenia congénita, Medicina, Buenos Aires **4**: 46, 1943.
   (h) Henderson, J. L., Donaldson, G. M. M., and Scarborough, H.: Congenital Afibrinogenaemia; Report of a Case With Review of the Literature, Quart. J. Med. **14**: 101, 1945.
3. (a) Risak, E.: Konstitution und haemorrhagische Diathesen, Wien. klin. Wchnschr. **47**: 1192, 1934.
   (b) Allibone, E. C., and Baar, H. S.: Fibrinogen Deficiency as a Factor in Haemorrhagic Disease, Arch. Dis. Childhood **18**: 146, 1943.
4. Opitz, H., and Silberberg, M.: Afibrinogenamie und Thrombopenie infolge aus gedehnter hepato-lienaler Tuberkulose, Klin. Wchnschr. **3**: 1443, 1924.
5. Knauer, H.: Das Krankheitsbild der Purpura fulminans, Jahrb. f. Kinderh. **118**: 1, 1928.
6. Jürgens, R., and Trautwein, H.: Ueber Fibrinopenie (Fibrinogenopenie) beim Erwachsenen, nebst Bemerkungen über die Herkunst des Fibrinogen, Deutsches Arch. f. klin. Med. **169**: 28, 1930.
7. (a) Risak, E.: Die Fibrinopenie, Ztschr. f. klin. Med. **128**: 605, 1935.
   (b) Waldenstrom, J.: Incipient Myelomatosis or "Essential" Hyperglobulinemia With Fibrinogenopenia—a New Syndrome? Acta med. Scandinav. **117**: 216, 1944.
8. Dieckmann, W. J.: Blood Chemistry and Renal Function in Abruptio Placentae, Am. J. Obst. & Gynec. **31**: 734, 1936.
9. Field, J. B., and Dam, H.: Influence of Diet on Plasma Fibrinogen in the Chick, J. Nutrition **31**: 509, 1946.
10. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Baltimore, 1931, Williams & Wilkins Company.
11. (a) Goetz, R. H.: The Pathology of Progressive Systemic Sclerosis (Generalized Scleroderma) With Special Reference to Changes in Viscera, Clin. Proc. **4**: 337, 1945.
   (b) Pugh, D. G., Krale, W. F., and Margulies, H.: Scleroderma With Involvement of the Viscera; Report of a Case, Proc. Staff Meet., Mayo Clin. **20**: 410, 1945.
12. Schmidt, C. R., Greengard, H., and Ivy, A. C.: Comparison of Methods for Quantitative Estimation of Diastase in Duodenal Fluid, Am. J. Digest. Dis. **1**: 618, 1934.
13. Hawk, P. B., and Bergeim, O.: Practical Physiological Chemistry, ed. 11, Philadelphia, The Blakiston Company, pp. 453.
14. Kato, K.: Microprothrombin Test With Capillary Whole Blood, Am. J. Clin. Path. **10**: 147, 1940.
15. Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P.: Simple Bedside Test for Control of Vitamin K Therapy, Am. J. Clin. Path., Tech. Supp. **4**: 13, 1940.

BIOCHEMICAL STUDIES DURING MALARIAL AND ARTIFICIAL FEVERS

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THERE have been numerous inquiries into the metabolic alterations produced by fever.[1-10] Recent communications, however, reveal a diversity of opinion[9, 11, 12] and a surprising paucity of observations made during the actual course of a febrile episode. Despite the current universal interest in malaria and the number of investigations directed at this disease, there are few precise data available regarding variations produced in the chemical constituents of the blood.

We were accidentally led into the study of this subject as the result of an observation on a patient who developed tetany during the course of an attack of recurrent malaria. There was present in this case neither hypocalcemia nor alkalosis but instead a profound degree of hypophosphatemia (serum inorganic phosphorus, 0.9 mg. per cent). Since this depletion of phosphorus was found to be transitory, the serum phosphorus having returned to 3.2 mg. per cent within twenty-four hours, it was thought that investigation of other patients with malaria might reveal similar findings. Accordingly, serum phosphorus estimations were made in eighteen additional cases during or within two hours after a chill. None of these showed evidence of tetany. Among these, fourteen patients exhibited serum phosphorus levels between 1.5 and 3.0 mg. per cent. In the remaining four the phosphorus level was normal. It was noted, however, that in those instances in which the serum phosphorus had been normal or at the low borderline level (3.0 mg. per cent) blood samples had been procured rather early in the chill.

Although hypophosphatemia had beeen described previously in association with the malarial paroxysm,[9] it appeared that no accurate relationship had been established for its time of occurrence or duration. Moreover, there was no conformance in opinions[11-13] regarding the changes effected in other blood constituents. It was decided, therefore, to carry out a study under controlled circumstances and further to determine if possible the existence of parallel changes in nonmalarial fever.

MATERIALS AND METHODS

The study was carried out on three groups of patients as follows:

Group A.—Serum phosphorus levels were determined at serial intervals during sixteen febrile paroxysms in twelve patients with *Plasmodium vivax* malaria. The time of onset of the chill was noted and its relationship to the procurement of the various blood samples established (Table I).

From The Valley Forge General Hospital, Phoenixville, Pa.
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TABLE I. SERUM INORGANIC PHOSPHORUS IN RELATION TO ONSET OF MALARIAL PAROXYSM

PATIENT	SERUM PHOSPHORUS AT CHILL ONSET (MG. %)	SERUM PHOSPHORUS 1 HR. AFTER CHILL (MG. %)	SERUM PHOSPHORUS 2 HR. AFTER CHILL (MG. %)	SERUM PHOSPHORUS 3 HR. AFTER CHILL (MG. %)	SERUM PHOSPHORUS 4 HR. AFTER CHILL (MG. %)	SERUM PHOSPHORUS 5 TO 8 HR. AFTER CHILL (MG. %)	SERUM PHOSPHORUS 24 TO 72 HR. AFTER CHILL (MG. %)
W. J. H. (a)*	---	4.1	---	1.3	1.2	1.6	3.6
W. J. H. (b)*	3.2	2.7	---	---	---	---	---
L. F.	---	3.2	2.8	3.0	---	---	4.1
J. G. B.	---	2.4	1.2	---	2.0	3.5	3.9
J. P. W.	---	1.3	2.4	2.8	3.1	---	3.1
W. S.	2.9	2.2	2.9	3.0	3.4	1.8	3.0
R. G. S.	3.2	---	1.7	---	---	---	3.6
R. E. S. (a)	3.0	---	---	2.5	---	3.6	---
R. E. S. (b)	---	2.3	---	---	---	2.9	3.0
R. W. S.	4.2	---	2.0	---	---	---	3.7
H. D. P.	---	---	3.0	3.2	3.3	3.4	---
A. M. (a)	---	---	1.8	2.4	2.0	---	---
A. M. (b)	2.5	---	1.4	1.0	---	---	---
T. L. J. (a)	---	---	3.1	2.1	2.1	---	3.0
T. L. J. (b)	---	2.8	2.2	1.6	---	---	---
P. H.	3.4	2.3	2.0	1.8	---	---	4.2

*(a) and (b) refer to two successive paroxysms in the same patient without therapy.

Group B.—Fifteen patients with benign tertian malaria were selected with a view to excluding complicating diseases or injuries which might in some way influence observations. The studies were instituted within a relatively short period after the onset of the chill and were carried out serially until the temperature fell. Therapy or food were withheld throughout the period of observation, the patients receiving neither antimalarial nor antipyretic drugs. Water was permitted as desired. Records were kept of the temperature changes and their relationships to the time of collection. Both clotted and nonclotted samples were obtained. The anticoagulant employed in the latter consisted of sodium fluoride and mercuric chloride designed to inhibit glycolysis. Spectrophotometric analyses of the following substances were performed: serum inorganic phosphorus,[14, 15] serum calcium,[16] serum magnesium,[17] serum potassium,[18, 19] blood sugar,[20, 21] and total blood-reducing substances.[22] In addition to the afore-mentioned tests, serum protein determinations[23] also were carried out.

The results of these analyses are shown in Table II. Two additional patients (Cases XVI and XVII) were subsequently studied to determine if there was any relationship between the hypophosphatemia and the blood creatine and creatinine (Table III). Observations were carried out under the same conditions as previously outlined.

Group C.—This group consisted of ten patients subjected to artificial fever therapy. Seven of these with minor eye ailments were treated in a fever cabinet in which the temperature was raised to 104° F. (rectal) and maintained for one and one-half hours. The other three patients with paresis, were treated in a similar apparatus in which body temperature was raised to 106° F. (rectal) and maintained at this level for four hours. Fasting blood samples were obtained before the patients entered the fever cabinet and temperatures were recorded continuously by means of a thermocouple thermometer. Blood samples were obtained at intervals during the rise and fall of temperature as in Group B. The seven patients with eye disorders received only a liter of water per os during the course of the treatment and no preliminary medication. They were in a fasting state. The three patients with paresis also were in a fasting state, but they were given 200 mg. of ascorbic acid by mouth before entering the cabinet and while febrile were permitted a liter of 2 per cent saline solution.* The results in this group are summarized in Table IV.

*This was the routine at the Norristown State Hospital.

TABLE II.—BIOCHEMICAL STUDIES IN PATIENTS WITH BENIGN TERTIAN MALARIA

CASE	TIME* (HR.: MIN.)	TEMPERATURE (° F.)	SERUM PO_4 (MG. %)	SERUM Ca (MG. %)	SERUM Mg (MG. %)	SERUM K (MG. %)	TOTAL PROTEIN (%)	BLOOD GLUCOSE (MG. %)	REDUCING SUBSTANCE† (MG. %)
I	0:20	101	3.2	10.8	1.6	23	5.8	156	19
	2:00	102	1.9	10.8	2.6	23	5.8	172	16
	3:00	101	1.5	11.1	1.6	21	5.8	220	--
	6:00	101	1.8	11.0	2.6	19	5.1	216	8
	7:00	100.4	2.5	11.0	3.3	20	5.1	156	8
	8:00	99.6	3.1	11.0	2.8	19	5.1	152	12
	12:00	98	3.9	11.1	1.7	21	5.1	123	11
II	1:20	101	3.5	10.6	2.3	20	6.2	120	12
	2:00	104.4	2.5	10.6	2.3	18	5.4	134	10
	2:30	104.6	2.3	10.6	2.0	19	5.8	164	10
	3:30	105.2	1.9	10.8	2.3	18	5.8	178	28
	4:15	103.2	1.7	10.9	2.3	18	5.5	172	24
	6:00	102.5	1.9	10.8	1.8	29	5.8	168	6
	8:15	100.6	3.5	10.8	1.9	32	5.8	197	8
	9:00	98.8	5.4	11.0	1.8	28	5.8	156	12
III	0:10	101.6	4.7	11.1	2.3	23	6.2	124	14
	0:40	103.8	4.6	11.1	2.3	21	6.6	132	4
	1:10	104.6	3.8	11.1	3.2	20	6.5	138	4
	1:40	106.4	3.2	11.3	3.0	22	6.2	140	16
	2:20	106.4	2.4	11.1	1.8	20	5.5	163	--
	3:00	105.8	2.2	11.1	2.3	20	5.6	146	--
	5:50	104.8	1.5	10.6	3.0	20	5.5	148	--
	8:00	102.0	2.0	10.7	2.3	29	5.1	154	12
	9:00	101.0	3.0	10.4	2.3	29	5.5	164	13
	10:20	100.0	4.4	10.4	3.2	28	5.1	149	13
	13:00	98.2	4.7	10.6	2.8	30	5.4	172	12
IV	0:10	101.0	3.6	10.7	--	--	6.5	87	--
	1:00	105.2	2.9	10.2	--	--	6.6	130	--
	2:00	105.0	2.5	10.0	--	--	6.5	168	--
	3:00	105.0	1.9	9.8	--	--	6.5	170	--
	10:00	100.0	1.2	9.1	--	--	6.7	176	--
	18:15	100.0	1.7	9.8	--	--	6.5	158	--
V	0:10	97.0	3.8	10.3	--	--	6.2	110	--
	0:35	--	3.2	10.6	--	--	5.7	123	--
	1:35	103.6	2.5	10.5	--	--	5.7	129	--
	2:35	104.6	2.2	9.4	--	--	5.5	138	--
	3:35	104.2	--	--	--	--	--	--	--
VI	0:40	103.6	4.3	9.6	--	--	--	121	--
	1:30	103.6	2.6	9.8	--	--	--	106	--
	2:30	104.3	1.9	9.8	--	--	6.4	192	--
	3:30	104.0	1.8	10.2	--	--	--	220	--
	13:30	100.0	3.4	9.8	--	--	--	132	--
VII	0:30	103.2	3.1	11.5	4.9	21	--	154	--
	1:30	104.0	3.9	11.5	4.8	20	--	134	--
	2:30	103.2	3.0	--	4.7	21	--	126	--
	5:00	104.6	2.9	11.5	5.2	21	--	158	--
	7:00	103.6	3.1	11.4	5.1	19	--	134	--
	8:00	103.4	2.7	11.5	--	22	--	114	--
	12:30	105.0	2.5	11.6	4.4	23	--	142	--
	24:00	98.0	4.9	10.8	4.9	21	--	146	--
VIII	0:30	102.6	3.6	11.5	4.6	22	--	116	12
	1:30	104.4	3.2	11.6	3.3	18	--	145	10
	2:30	104.4	2.8	11.4	3.0	19	--	146	8
	3:30	104.2	2.7	11.3	3.0	21	--	130	5
	4:30	102.8	3.1	11.4	4.7	19	--	130	8
	5:30	103.0	3.9	11.4	4.9	--	--	130	12
	9:00	100.0	4.7	11.4	2.0	17	--	120	8
	11:10	98.8	4.4	11.3	2.2	17	--	112	8

TABLE II—CONT'D

CASE	TIME* (HR.: MIN.)	TEMPERATURE (° F.)	SERUM PO_4 (MG. %)	SERUM Ca (MG. %)	SERUM Mg (MG. %)	SERUM K (MG. %)	TOTAL PROTEIN (%)	BLOOD GLUCOSE (MG. %)	REDUCING SUBSTANCE† (MG. %)
IX	0:20	99.6	2.7	10.9	3.1	--	--	130	12
	1:30	103.0	1.5	11.2	4.5	--	--	126	11
	2:30	104.0	0.8	11.0	2.5	21	--	170	6
	3:30	103.6	1.1	11.2	3.2	19	--	152	3
	4:30	102.0	1.6	11.1	2.4	21	--	134	4
	5:30	101.4	2.4	11.3	2.7	17	--	156	7
	8:00	99.4	4.3	11.3	3.9	18	--	133	10
X	0:10	99.0	4.5	9.3	--	--	6.8	98	--
	1:00	102.6	2.8	10.3	--	--	6.5	110	--
	2:00	105.6	2.2	9.7	--	--	6.0	138	--
	3:00	105.6	1.8	10.5	--	--	6.0	142	--
	4:00	105.2	3.9	10.4	--	--	6.0	158	--
	6:30	97.6	4.2	10.5	--	--	6.2	179	--
	10:30	97.6	3.6	10.6	--	--	6.9	184	--
XI	1:00	103.0	2.5	10.1	2.1	19	6.8	132	--
	1:15	105.0	2.1	10.3	1.8	18	6.5	136	--
	1:40	104.6	1.9	10.3	3.6	18	6.2	146	--
	2:40	105.0	1.4	10.3	2.3	17	5.8	146	--
	3:00	104.0	1.1	10.3	1.9	19	5.8	144	--
	4:25	103.2	1.6	10.5	2.4	16	5.8	149	--
	5:30	101.4	3.3	10.5	1.9	24	6.2	140	12
	13:15	99.6	4.3	10.3	1.9	26	6.2	124	13
	20:30	98.0	3.7	10.3	1.8	18	6.2	113	13
XII	0:10	97.0	5.3	11.1	5.0	21	--	118	13
	0:45	104.2	3.9	10.9	2.7	22	--	139	10
	1:30	103.0	2.8	10.7	3.2	21	--	160	7
	2:15	102.0	1.9	10.8	2.1	22	--	141	7
	6:05	101.0	3.9	11.2	2.4	22	--	130	8
	6:30	100.0	4.4	11.2	4.0	18	--	136	9
	8:45	99.0	4.8	11.0	3.5	18	--	138	12
	11:45	98.0	5.1	11.1	2.1	18	--	120	7
XIII	1:50	99.6	1.2	10.6	3.0	17	5.9	152	8
	3:00	103.4	3.3	10.7	1.4	16	5.9	130	4
	4:00	103.0	--	10.7	--	--	--	146	--
	4:45	102.4	3.1	10.8	1.6	19	5.9	176	4
	6:50	99.4	4.2	10.8	1.6	18	5.9	196	2
	17:15	98.2	3.8	10.8	2.0	20	5.7	126	2
XIV	0:30	101.8	2.1	10.5	2.2	18	6.0	168	20
	2:00	104.0	1.5	10.5	2.3	15	5.8	190	17
	3:00	104.6	1.1	10.7	3.7	15	5.5	170	12
	4:00	105.2	0.9	10.4	5.5	--	5.1	170	14
	7:00	100.2	3.4	11.1	3.9	22	5.5	220	18
	20:00	98.2	4.1	10.5	5.3	30	7.2	220	14
XV	0:20	104.4	2.0	9.8	1.6	19	6.2	140	6
	1:30	105.2	1.7	10.8	3.0	16	5.8	220	6
	2:40	104.6	1.3	10.6	2.0	17	5.9	250	10
	4:30	103.8	1.9	10.9	2.7	16	5.9	236	8
	5:45	103.2	2.9	10.9	1.3	19	5.9	196	2
	7:55	99.6	4.1	10.8	1.4	17	5.9	212	14
	9:10	99.0	4.9	10.8	3.0	21	5.9	168	5
	11:30	98.6	4.9	10.5	1.7	21	5.9	168	6

*Interval following the onset of the chill.

†Total reducing substances in blood other than glucose; results expressed in terms of milligrams per cent of glucose.

TABLE III. BLOOD CREATINE AND CREATININE STUDIES DURING MALARIAL PAROXYSMS

CASE	TIME* (HR.:MIN.)	TEMPERATURE (° F.)	SERUM PO_4 (MG. %)	BLOOD GLUCOSE (MG. %)	BLOOD CREATININE (MG. %)	BLOOD CREATINE (MG. %)	SERUM PROTEIN (%)
XVI	0:10	99	2.5	106	1.5	5.7	5.8
	0:30	103.8	2.3	106	1.6	6.0	5.5
	0:45	105.6	2.1	126	1.5	5.2	6.0
	1:30	105.0	2.5	154	1.5	5.7	5.5
	2:30	105.3	1.3	148	1.5	5.5	5.8
	3:40	105.0	0.8	154	1.5	5.0	5.5
	5:40	104.3	1.0	154	1.6	4.9	5.8
	6:40	102.3	1.9	154	1.7	5.0	6.2
	7:40	99.8	3.6	154	1.8	5.3	5.5
	23:00	97.0	3.8	122	1.6	5.3	6.2
XVII	0:10	99	2.6	122	1.4	6.6	6.5
	0:50	103.6	2.4	144	1.5	5.7	6.5
	1:20	104.6	1.9	162	1.3	6.3	5.8
	2:50	104	1.7	178	1.4	6.6	6.0
	5:50	103	1.9	132	1.4	5.8	6.0
	7:20	102.2	2.9	90	1.4	5.8	6.5
	9:30	104.0	3.0	136	1.4	5.8	6.5
	12:00	101.2	3.6	150	1.5	6.1	6.5

*Interval following the onset of the chill.

TABLE IV. BIOCHEMICAL STUDIES IN PATIENTS WITH ARTIFICIALLY INDUCED FEVER

CASE	TIME* (HR.: MIN.)	TEMPERATURE (° F.)	SERUM PO_4 (MG. %)	SERUM Ca (MG. %)	SERUM Mg (MG. %)	SERUM K (MG. %)	TOTAL PROTEIN (%)	BLOOD GLUCOSE (MG. %)	REDUCING SUBSTANCE† (MG. %)
I	0:00	98.6	4.5	9.8	--	--	7.3	92	--
	1:00	102.0	4.1	9.6	--	--	7.1	116	--
	1:15	104.0	3.9	9.8	--	--	6.8	126	--
	2:35	104.0	3.3	9.6	--	--	6.7	128	--
	3:30	102.0	2.8	9.6	--	--	6.7	140	--
	3:45	99.0	3.2	9.2	--	--	7.1	163	--
II	0:00	97.6	2.1	10.4	--	--	7.2	118	--
	1:10	102.0	1.5	10.5	--	--	7.4	106	--
	1:45	103.2	1.1	11.0	--	--	7.8	130	--
	3:00	103.2	0.7	11.7	--	--	7.6	134	--
	3:45	99.0	0.9	10.6	--	--	7.2	134	--
III	0:00	99.4	3.5	10.9	--	--	6.5	109	--
	0:40	102.0	4.1	10.9	--	--	6.5	106	--
	1:35	104.0	3.8	10.9	--	--	6.5	108	--
	3:00	104.0	3.7	10.8	--	--	6.7	108	--
	3:20	99.2	3.2	10.9	--	--	6.5	102	--
IV	0:00	97.0	3.7	10.9	--	21	6.6	175	--
	1:00	102.0	3.1	10.9	--	20	6.6	170	--
	1:50	104.0	2.5	10.9	--	18	6.6	188	--
	3:15	104.2	2.1	10.9	--	18	7.1	197	--
	3:35	102.0	2.2	11.0	--	17	6.5	294	--
	3:45	98.8	2.6	11.0	--	18	6.5	276	--
V	0:00	98.2	4.1	10.8	1.4	21	6.3	120	--
	0:40	102.0	3.5	11.0	1.6	24	6.5	132	--
	1:10	105.0	2.6	11.5	2.2	24	6.3	136	--
	3:00	104.7	1.7	11.5	1.8	21	6.6	205	--
	3:15	102.0	1.9	11.1	1.2	19	6.3	188	--
	3:30	99.0	2.2	11.1	1.3	20	6.3	176	--

TABLE IV—CONT'D

CASE	TIME* (HR.: MIN.)	TEMPERATURE (° F.)	SERUM PO_4 (MG. %)	SERUM Ca (MG. %)	SERUM Mg (MG. %)	SERUM K (MG. %)	TOTAL PROTEIN (%)	BLOOD GLUCOSE (MG. %)	REDUCING SUBSTANCE† (MG. %)
VI	0:00	98.6	4.4	10.4	2.0	18	--	81	15
	0:55	102.0	4.0	10.1	2.4	20	--	148	29
	1:15	104.4	3.8	10.1	1.8	22	--	130	11
	2:15	104.7	3.3	10.7	3.8	19	--	138	11
	3:30	102.2	4.1	10.7	1.9	19	--	146	15
	3:45	98.0	3.6	10.5	1.8	19	--	154	11
VII	0:00	97.0	4.9	11.1	3.6	--	--	150	6
	1:10	102.0	4.0	11.0	2.3	21	--	120	14
	1:30	104.2	3.4	11.3	2.0	23	--	123	3
	3:00	104.3	2.6	11.2	1.6	22	--	166	5
	3:45	102.0	2.6	11.1	1.9	21	--	166	4
	4:00	99.6	2.8	11.1	1.7	20	--	149	4
VIII	0:00	97.6	3.9	9.9	2.6	--	--	98	23
	0:45	100.0	4.1	9.5	1.5	--	--	112	14
	1:00	102.0	3.4	9.6	2.7	--	--	112	10
	1:45	106.0	2.7	9.6	2.6	--	--	124	20
	3:45	106.0	1.8	9.8	3.6	--	--	126	14
	5:45	106.0	2.2	10.0	4.0	--	--	128	29
	6:15	104.0	2.5	10.5	5.5	--	--	138	24
	6:30	102.0	2.9	10.2	4.0	--	--	128	16
	7:00	100.0	4.7	10.1	4.0	--	--	116	10
	8:05	99.2	4.9	10.5	4.0	--	--	112	6
IX	0:00	98.2	5.0	9.0	4.2	21	6.2	120	10
	0:25	100.0	4.7	10.2	2.8	18	6.2	122	15
	0:50	102.0	3.9	10.2	1.7	17	6.1	126	8
	1:00	104.0	3.6	10.2	2.0	20	6.5	131	12
	1:20	106.0	3.1	10.3	1.9	20	6.5	131	12
	2:20	106.0	3.3	10.4	1.8	18	6.3	132	13
	3:20	106.4	3.2	10.0	2.2	--	6.5	139	8
	4:20	106.2	3.4	9.5	1.9	18	6.6	156	10
	5:20	105.3	3.6	9.3	2.5	16	6.6	153	16
	5:40	102.0	4.1	10.3	1.8	16	6.1	156	11
	6:10	100.2	4.6	10.0	2.4	17	5.8	138	14
X	0:10	98.4	2.9	10.5	2.8	33	7.2	214	30
	0:30	100.0	2.6	10.5	3.8	31	7.2	123	10
	0:40	102.0	2.2	10.3	4.1	32	7.0	144	14
	1:15	104.0	1.9	10.5	--	--	6.9	134	10
	1:40	106.0	2.2	10.6	2.3	34	6.8	198	10
	3:40	106.0	1.9	10.6	3.9	36	6.8	132	6
	5:40	106.0	1.7	10.5	4.7	33	6.9	200	10
	6:05	104.0	2.1	10.7	3.7	31	7.5	192	8
	6:12	102.0	2.3	10.7	3.0	--	7.5	198	11
	6:40	100.0	3.0	10.6	5.7	--	--	184	21
	7:10	99.6	3.8	10.7	4.4	33	7.2	173	10

*Interval following the onset of the chill.

†Total reducing substance in the blood other than glucose; results expressed in terms of milligrams per cent of glucose.

RESULTS

Group A.—In fifteen of the sixteen paroxysms studied in this group the serum phosphorus level fell below the limit of normal (3.0 mg. per cent). The average minimum level attained was 1.9 mg. per cent with a range from 1.0 to 2.9 mg. per cent. The reduction in serum phosphorus was found to be progressive and minimum levels were not reached until some time after the inception of the rigor (Table I).

Group B.—As shown in Tables II and III, hypophosphatemia occurred in all seventeen of these patients. The average minimum level reached was 1.6 mg. per cent with a range from 0.8 to 2.7 mg. per cent. Among fourteen cases in which phosphorus levels were analyzed very early in the chill before a significant fall had occurred, the total diminution in this mineral averaged 51 per cent of the original level (range 19 to 71 per cent).

As surmised, it was found that the development of hypophosphatemia was progressive, commencing at the time of the temperature rise with the minimum level occurring after about three or four hours. Here, however, the range was wide (two to twelve hours). If the fever and serum phosphorus levels were plotted graphically (Figs. 1*A* and 1*B*), however, it was apparent that there was an inverse relationship between the two, the latter attaining its lowest level at about the time of the fever peak. Thereafter, however, the hypophosphatemia disappeared fairly rapidly despite a continued elevation or even a secondary rise in temperature. Normal blood levels were restored before the fever had wholly subsided.

Of the other blood constituents studied only glucose exhibited a consistent relationship to the changes previously noted. Since the metabolic insult of the parasitemia preceded the inception of rigor, none of the patients could be considered to be in a basal state. It was not possible, therefore, to establish a basal take-off point in this group. Nevertheless, all of the patients exhibited an elevation of blood glucose as the febrile episode proceeded. There was an obvious tendency for the glucose level to rise parallel with the fever, but this rise continued for a number of hours after the pyrexia had arrived at its peak and had begun to subside (Figs. 1*A* and 1*B*). The maximum blood sugar levels attained averaged 184 mg. per cent with a range of 138 to 250 mg. per cent. Seven cases showed values exceeding the average. From twelve of these patients urine samples were procured during and after the paroxysm, and despite the fact that in seven of them the usual renal threshold level of 180 mg. per cent was exceeded no glycosuria was detected.

In ten of the cases fractionation of blood-reducing substance was carried out. Some variation of the nonglucose fraction occurred but was inadequate in amount to account for the rise in total reducing substance. It was apparent, therefore, that the blood sugar rise observed was almost wholly the result of increase in glucose.

Despite the very significant variation in the concentration of serum phosphorus, the calcium levels remained relatively unchanged throughout the paroxysm. At times mild hypocalcemia was noted in the earlier cases sub-

jected to preliminary studies, but in none of the rigidly selected individuals was this noted. The variation in values from the inception to the termination of the episodes was a fraction of a milligram.

Serum potassium evaluations were carried out in eleven instances. Although variation did appear the values generally remained within the limits of normal (18 to 22 mg. per cent). In two cases a potassium rise to 30 and 32 mg. per cent, respectively, occurred during the period of temperature fall, but this phenomenon was not observed in the remaining nine patients.

The value of serum magnesium was determined serially in eleven patients. As with the serum potassium, such variations as were observed were usually confined within the limits of normal (1 to 3 mg. per cent). In some instances normal values were exceeded but there was no consistent relationship to the fever or other metabolites.

In ten cases total protein estimations were made. In two of these patients the protein remained unchanged. In the remaining eight an average fall of 0.8 Gm. occurred during the paroxysm (0.4 to 1.5 Gm.). In most instances a rising trend or a return to pre-existing values had appeared at the termination of the study.

In two cases blood creatine and creatinine determinations were performed. With the latter the greatest change was 0.3 mg. per cent, while with the former there was a reduction of 1 mg. at some time during the temperature rise. This alteration of about 17 per cent was transitory and values of blood creatine remained within the limits of normal (Table III).

Group C.—The chemical studies carried out in this group were identical with those in Group B. The results, likewise, were somewhat similar. Among these patients the serum phosphorus levels exhibited a progressive fall as the fever curve rose (Figs. 2*A* and 2*B*), although the degree of change was not quite as great. The minimum phosphorus levels ranged from 0.7 to 3.3 mg. per cent, the average being 2.3 mg. per cent. The average fall from the pretreatment level was 41 per cent and the range from 9 to 67 per cent. It appeared that the minimal level was reached somewhat more rapidly than in those cases with malarial pyrexia (Figs. 2*A* and 2*B*), the average time being two or three hours after the induction of fever. However, the return to within normal phosphorus limits was more rapid than in the patients with malarial fever. Despite the greater rapidity of phosphorus fall in this group, the rate of temperature rise was greater so that minimum phosphorus levels occurred with a lag of one or two hours after the temperature had reached its peak.

Although these patients were all in a fasting state at the onset of the fever therapy, many showed an elevated blood sugar in the initial pretreatment sample. This may have been on a psychomotor basis as many of the patients manifested apprehension on entering the fever cabinet. One patient with a fasting blood sugar level of 175 mg. per cent showed a subsequent rise to 294 mg. per cent when the body temperature was elevated. Later investigation revealed that this patient suffered from a previously unsuspected diabetes mellitus. Of the remaining nine patients all but one showed a hyperglycemia similar to that observed with malarial fever, the rise in blood sugar roughly approximating

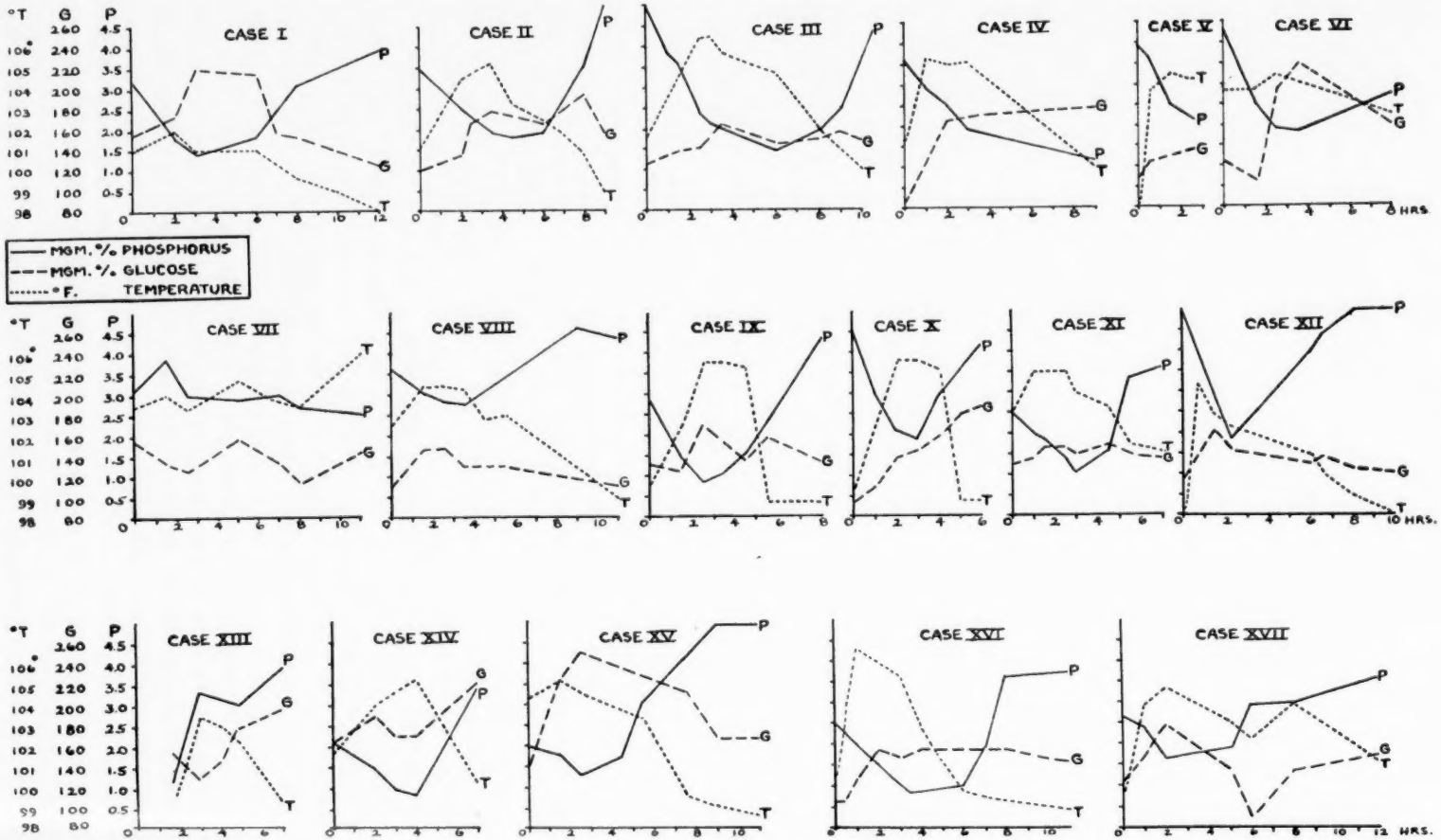

Fig. 14.—Glucose and phosphorus curves in malaria.

Fig. 14.—Glucose and phosphorus curves in malaria.

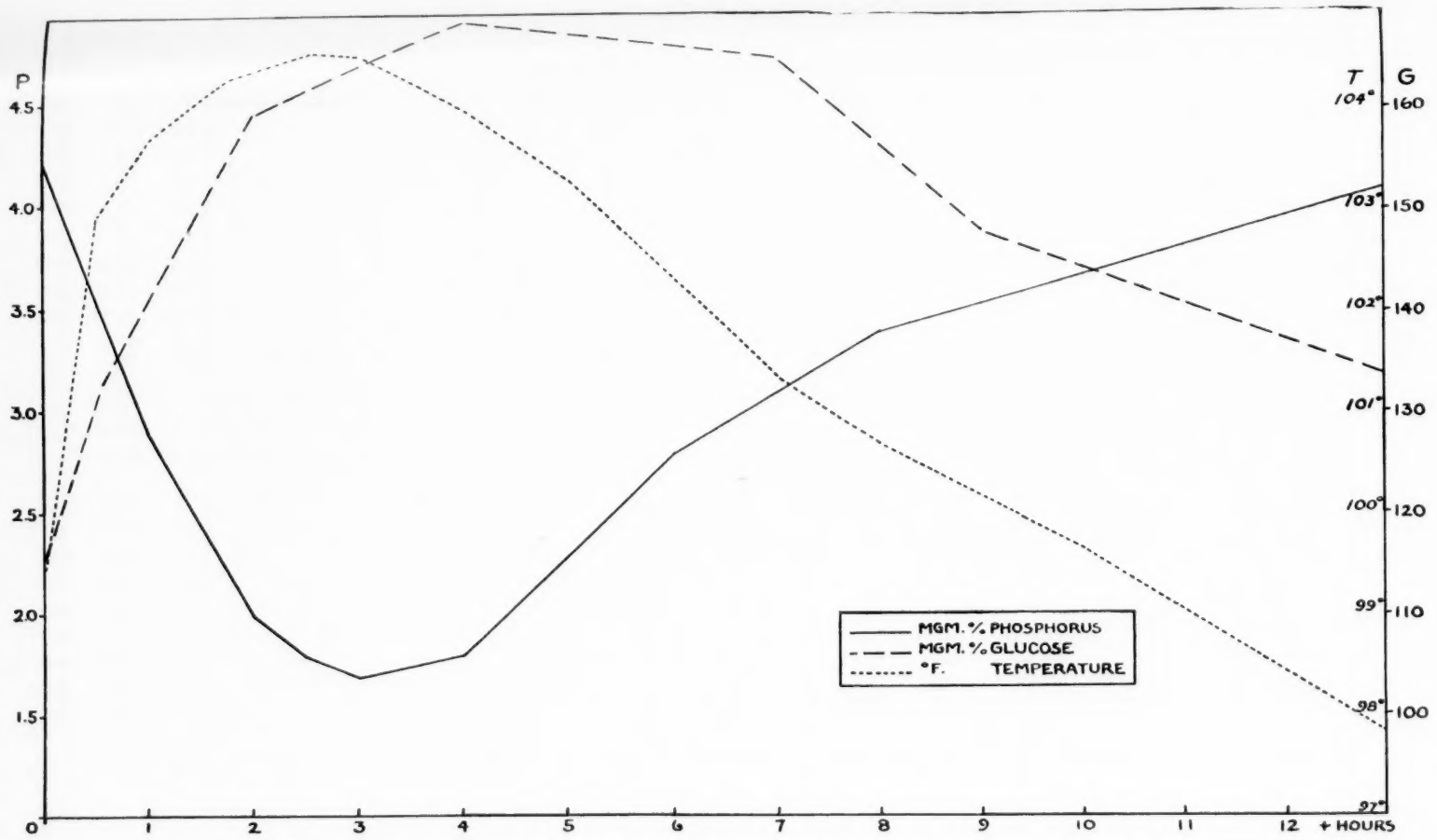

Fig. 1*B*.—Mean temperature and sugar and phosphorus curves during malaria paroxysm (ten cases).

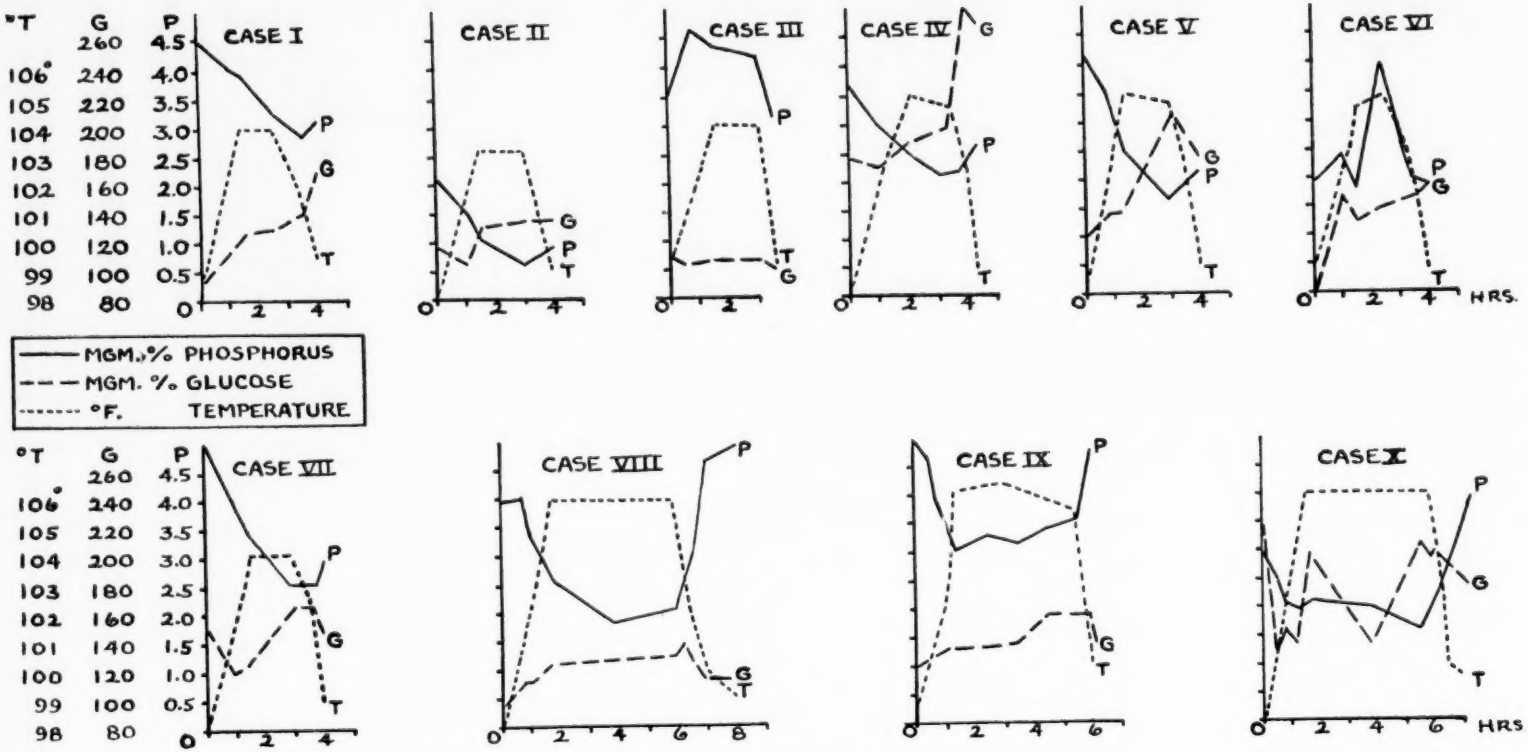

Fig. 24.—Glucose and phosphorus curves in artificially induced fever.

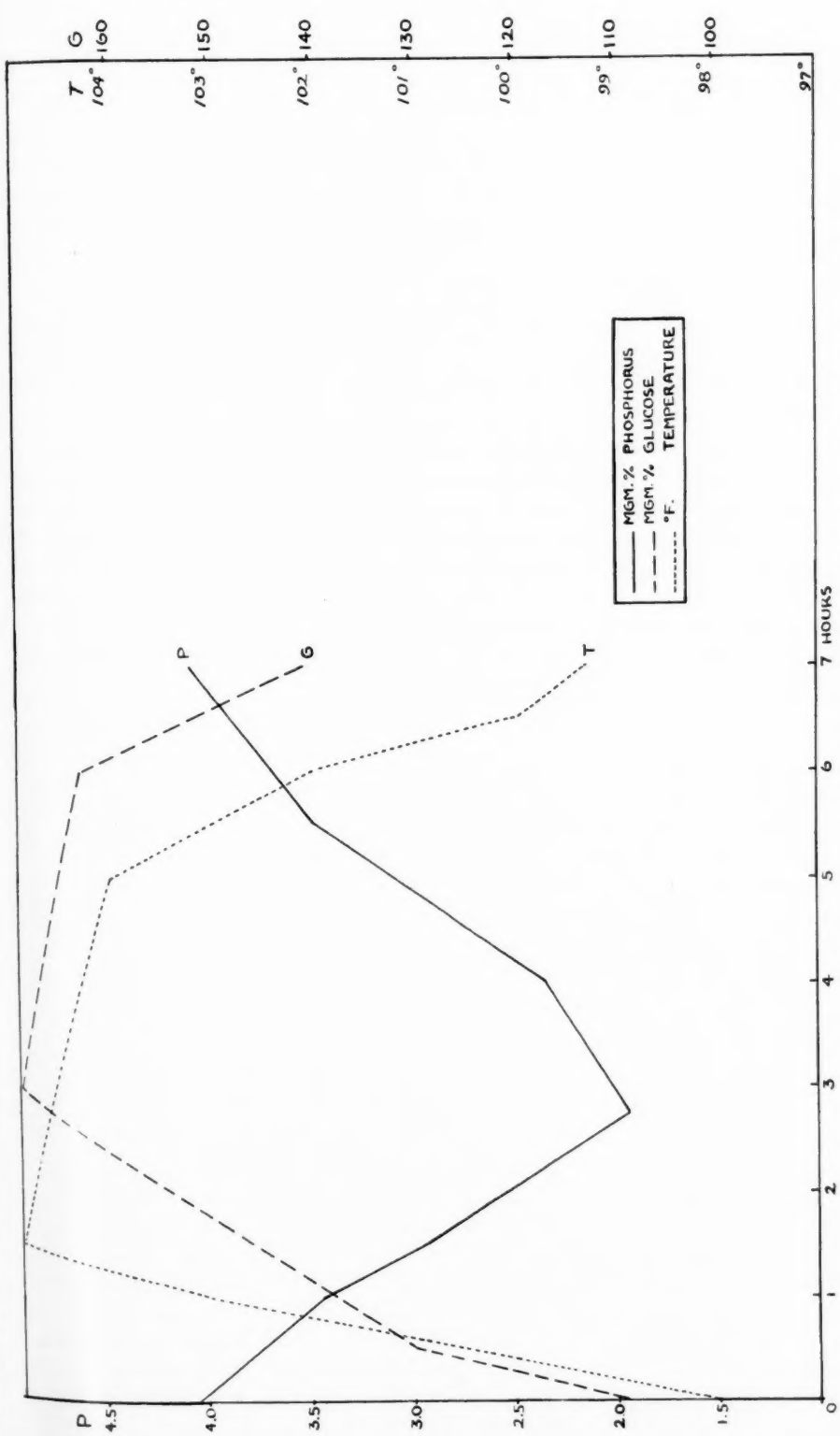

Fig. 2B.—Mean temperature and sugar and phosphorus curves during artificially induced fever (ten cases).

the slope of the fever curve. As in the malarial group, the rise continued and the elevation persisted for a considerable period after the temperature had returned to normal.

Exclusive of the patient with diabetes (Case IV) and the patient showing no change in blood sugar (Case III), the range of maximum levels attained was 134 to 205 mg. per cent and the average was 165 mg. per cent. Four individuals from whom urine samples were obtained exhibited no glycosuria. In two of these the blood sugar level had exceeded 180 mg. per cent.

The blood from five of these patients was analyzed for nonglucose-reducing substance and, as noted previously, such variation as occurred was inadequate to account for the rise in total reducing substance. The latter therefore consisted in the main of glucose.

Alterations in serum calcium and serum magnesium were of the order previously noted in the malarial studies. The amount and kind of variation was such as to preclude importance in the metabolic processes involved. Although one patient with paresis (Case X) showed a persistent elevation of serum potassium throughout the entire study, the fluctuation in the amount of this element in the other cases was considered insignificant. The total serum protein estimations, likewise, were of little moment. In one case there was no change, in four cases an increase of 0.4 to 0.6 Gm. per cent was noted, and in two cases there was a fall of 0.4 to 0.6 Gm. per cent.

DISCUSSION

A reduction in serum inorganic phosphorus was observed initially in a group of patients with malaria. The phenomenon subsequently was shown to begin toward the end of a chill when the rise in temperature was initiated and to decline to the minimum point when the fever had attained its peak. Thereafter, the phosphorus values returned rapidly to normal limits often without the reciprocal relationship to the temperature curve originally shown. The occurrence of hypophosphatemia in association with the malarial paroxysm had been noted earlier by Payne.[9] A similar reduction in phosphorus observed by us in a series of patients receiving artificial fever therapy paralleled those recorded previously by other investigators.[4, 5, 10, 24, 50]

In seeking an explanation for the hypophosphatemia, blood glucose determinations were made at comparable periods. From these studies it became apparent that the fall in serum phosphorus was attended by a rise in glucose in a manner which suggested an inverse relationship between the two. It appeared on the basis of chronological observations that the blood sugar rise occurred fairly promptly in the episode, often manifested in the initial samples procured. Return to normal values was prolonged as compared to the serum phosphorus. In many instances initial sugar values had not been re-established when the temperature reached normal. Similar observations were made during the induced febrile state. In many instances a secondary rise in temperature was attended by a further elevation in the blood sugar. However, there was no concomitant secondary reduction in serum phosphorus, the latter continuing

to approach the original normal levels without deviation. We have no explanation to offer for this paradox.

It was thought that the hyperglycemia thus produced resulted from an acceleration of metabolism[8, 25, 26] and the simultaneously increased muscular tonus.[9] It was suspected that the hypophosphatemia was linked with increased glucose utilization, a possibility further sustained by the reduction of serum phosphorus observed following the ingestion of glucose and/or the administration of insulin.[27-31]

It was postulated that the period characterized by the hypophosphatemia was associated with the process of phosphorylation. Glycogenolysis and glycogenesis occurring in the course of the febrile paroxysm probably were accompanied by the formation of hexosephosphate and the deposition of the latter in the blood and tissues.[32] The transient linkage of phosphorus with the carbohydrate component presumably resulted in hypophosphatemia. At the conclusion of the episode, the dissociation of hexosephosphate permitted the mobilization of phosphorus, the replenishing of inorganic phosphorus, and the concomitant rise of serum phosphorus to its prefebrile level. The hexose, it would seem, was reconverted thereafter to glycogen and deposited in the tissues or was decomposed further and excreted.

Some substantiation of this thesis is found in the report of Cori and Cori[33] who noted an increased amount of hexosephosphate in muscle during muscular activity and a disappearance of this compound when activity ceased. Of parallel interest was the recorded fall of blood sugar in diabetics following the parenteral administration of sodium phosphate.[34]

On the other hand, Payne[9] attributed the fall in serum phosphorus during induced malarial episodes primarily to the formation of phosphocreatine. This report was encountered after much of the present study was completed, but we were able to carry out similar estimations of creatine and creatinine values in two cases during spontaneous malarial chills (Table III). Blood samples were collected in these cases at frequent intervals after the onset of the chills, throughout the febrile course, and until the patients had become afebrile. We were unable to confirm Payne's observations of significant elevations in blood creatine at the time of the temperature rise. It is interesting that he had been unable to demonstrate any hyperglycemia during or following the paroxysm.

Although such urine phosphorus examinations as were carried out in our studies were crude, there is ample evidence in the literature attesting to the fact that loss of phosphorus does not occur through this medium during the febrile paroxysm.[4, 5, 9] Bischoff and associates[4, 5] attributed the hypophosphatemia and concomitant hyperglycemia encountered in experimental hyperthermia to the conversion of inorganic phosphorus to the organic state. Daly and Knudson,[24] however, were disinclined to accept this explanation since they noted diminution of both organic and inorganic phosphorus.

The hyperglycemia in artificial fever was considered by Hench and Slocumb[35] to be due to hemoconcentration. This thesis obviously is not correct in view of the concomitant hypophosphatemia and hyperglycemia and the evi-

dence of mild hemodilution shown by the diminished total protein values (Tables II to IV). Petersen[11] observed a diminution of blood sugar during the period of temperature rise in malaria with an elevation after the temperature had fallen. This discrepancy remains unexplained since it occurred in not a single one of the cases we observed. Kirstein and Bromberg[10] collected blood samples before induction and after subsidence of artificial fever. They studied no samples during the febrile period but recorded both increased glucose and diminished phosphorus after the hyperthermia. These authors considered the elevated sugar as evidence of increased glucose utilization by the tissues and also as basis for the serum phosphorus depletion. It seems reasonable to assume that results of a higher order of magnitude would have been obtained by Kirstein and Bromberg had they procured samples during the period of actual pyrexia.

Fenn,[36] reviewing the literature on potassium metabolism, remarked on the diminution of vascular tonus concomitant with a progressive loss of potassium and a rise in sodium during muscular activity. In our own studies of the serum potassium it did not appear that the level of this substance reflected the increased muscular activity during the rigor. For the most part the serum potassium varied only within normal limits. A few of the patients with malaria (Table II), as previously noted, showed an abrupt rise during the subsidence of the fever. One patient with paresis exhibited an inordinately high potassium level before fever was induced; this was not modified by the hyperpyrexia. None of these observations lend themselves to satisfactory explanation. A gradual elevation of serum potassium in malaria was reported by Zwemer and co-workers[37] and Stevenson,[38] the peak of rise being attained at the onset of the chill with a subsequent return to normal thereafter. Honaga[13] observed an increase of potassium in the blood at the height of the fever in several pathologic states with a return to normal during convalescence. On the basis of our own observations it did not appear that conclusions regarding the role of this electrolyte in fever were warranted.

The inverse relationship of serum magnesium to calcium and phosphorus and its action on the neuromuscular system[39-41] led to investigation of its variations in the febrile state. In both the spontaneous and induced fevers such variations as appeared, exhibited no consistent pattern. Peters and Van Slyke[42] have recognized this variability but were unable to offer an explanation for it. It is possible, as they suggested, that the methods for analysis used were sufficiently crude to permit such inconsistency. The magnesium values as we have observed them appear unrelated to the changes noted in other metabolites. For the most part their ranges were within normal limits.

Utilizing the amount of the total protein as an index of hydremia, it appeared that there was indeed some hemodilution of minor degree during the malarial paroxysm since the values of this substance fell rather uniformly at the febrile fastigium and rose again when the fever had subsided. Similar changes, however, were not noted in the patients with artificial fever. Certainly, there was no indication that the hyperglycemia noted was attributable to hemoconcentration as suggested by Simpson[43] and Hench and Slocumb.[35]

Studies upon other constituents of the blood revealed only incidental information. Determinations of the nonglucose-reducing substances concomitantly with the total blood-reducing substances showed that the rise in the latter was indeed the result of glucose per se and not one of the other associated compounds (ascorbic acid, cysteine, glutathione, uric acid). There was a remarkable constancy of serum calcium values which remained almost wholly unchanged throughout the febrile episode.

Studies with the cephalin-cholesterol flocculation[44] and thymol turbidity[45] tests were carried out simultaneously with those previously recorded. As had been noted previously,[46-48] the former were often positive in malaria. We obtained negative results in the artificial fever group. It is of interest to record that many of the patients with malaria showed positive flocculation and turbidity tests before and during the paroxysm. Others, however, showed a positive cephalin flocculation during the febrile period, the tests becoming negative when the temperature became normal. In these cases the thymol turbidity tests were negative throughout. The results of this phase of the study will be reported in detail subsequently in conjunction with a larger series of cases of miscellaneous nature.[49]

SUMMARY

1. Preliminary studies on patients with malarial fever (eighteen cases) revealed the development of transient hypophosphatemia during the febrile paroxysm.

2. Serum inorganic phosphorus, calcium, magnesium, potassium, serum protein, blood glucose, and nonglucose-reducing substance were determined at various intervals during the febrile episodes in patients with benign tertian malaria (seventeen cases).

3. Similar studies were carried out on patients with artificial fever therapy (ten cases).

4. There was a marked fall in serum phosphorus attended by a significant rise of the blood glucose in all patients with malaria and in the majority of fever therapy cases so studied.

5. It was believed that the formation and deposition of hexosephosphate in the tissues was the basis for the hypophosphatemia.

6. Hyperglycemia appeared early in fever and was subsequently followed by diminution in inorganic serum phosphorus.

7. No significant alterations were observed in the serum calcium, potassium, magnesium, creatine, or creatinine.

8. The serum protein showed a slight fall during the malarial fever but remained unchanged in artificial fever.

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REFERENCES

1. Beck, O.: Untersuchungen zum Fieberstoffwechsel, Klin. Wchnschr. **4**: 2276, 1925.
2. Cohn, H.: Experimentelle Hyperglykämie im Fieber, Ztschr. f. d. ges. exper. Med. **53**: 878, 1927.
3. Cohn, T., and Houget, J.: Le métabolisme des glucides, lipides, et protides au cours d'hyperthermies, Compt. rend. Soc. de biol. **104**: 852, 1930.
4. Bischoff, F., Ullmann, H. J., Hill, E., and Long, M. L.: Studies in Hyperthermia Induced by High Frequency Electric Current, J. Biol. Chem. **85**: 675, 1930.
5. Bischoff, F., Maxwell, L. C., and Hill, E.: The Acid-Base Equilibrium in Hyperthermia Induced by Short Radio Waves, J. Biol. Chem. **90**: 331, 1931.
6. Adachi, K.: Experimentelle Untersuchungen über den intermediären Stoffwechsel im Muskel bei Fieber; Phosphorsäure—und Harnsäurestoffwechsel im Muskel bei experimentellem Fieber, Tohoku J. Exper. Med. **20**: 107, 1932.
7. Grant, W. H., and Medes, G.: Creatinine Clearance During the Hyperthermia of Diathermy and Fevers, J. Lab. & Clin. Med. **20**: 345, 1934.
8. Kopp, I.: Metabolic Rates in Therapeutic Fevers, Am. J. M. Sc. **190**: 491, 1935.
9. Payne, W. W.: Phosphate and Creatine Metabolism in Fever, Biochem. J. **29**: 1310, 1935.
10. Kirstein, M. B., and Bromberg, L.: Effect of Fever Therapy on Carbohydrate Metabolism, J. Lab. & Clin. Med. **25**: 7, 1939.
11. Petersen, W. F.: Blood Sugar During Crisis of Malaria Fever, Proc. Soc. Exper. Biol. & Med. **23**: 753, 1926.
12. Emerson, K., Jr., and Dole, V. P.: Electrolyte, Water, and Nitrogen Metabolism in Acute P. vivax Malaria, Malaria Report No. 247, Sept. 22, 1944. (Board for the Coordination of Malarial Studies—*Restricted.*)
13. Honaga, S.: Clinical and Experimental Studies on the Metabolism of Calcium, Potassium, and Phosphorus During Fever, Jap. J. Exper. Med. **18**: 255, 1940.
14. Kuttner, T. T., and Cohen, H. R.: Micro Colorimetric Studies. I. A Molybdic Acid, Stannous Chloride Reagent; the Micro Estimation of Phosphate and Calcium in Pus, Plasma, and Spinal Fuid, J. Biol. Chem. **75**: 517, 1927.
15. Kuttner, T. T., and Lichtenstein, L.: Estimation of Phosphorus: Molybdic Acid—Stannous Chloride Reagent, J. Biol. Chem. **86**: 671, 1930.
16. Roe, J. H., and Kahn, B. S.: The Colorimetric Determination of Blood Calcium, J. Biol. Chem. **81**: 1, 1929.
17. Denis, W.: The Determination of Magnesium in Blood, Plasma, and Serum, J. Biol. Chem. **52**: 411, 1922.
18. Breh, F., and Gaebler, P. H.: The Determination of Potassium in Blood Serum, J. Biol. Chem. **87**: 81, 1930.
19. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Vol. 1, Baltimore, 1932, Williams & Wilkins Company, p. 753.
20. Folin, O., and Wu, H.: A Simplified and Improved Method for the Determination of Sugar, J. Biol. Chem. **41**: 367, 1920.
21. Folin, O.: A New Blood Sugar Method, J. Biol. Chem. **67**: 357, 1926.
22. Somogyi, M.: Reducing Non-Sugars and True Sugar in Human Blood, J. Biol. Chem. **75**: 33, 1927.
23. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M.: The Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma, Bumed News Letter of U. S. Navy, June, 1943.
24. Daly, C. A., and Knudson, A.: Acid-Base Equilibrium and Phosphorus Metabolism in Hyperthermia, J. Biol. Chem. **97**: 57, 1932.
25. Perera, G. A.: Clinical and Physiologic Characteristics of Chill, Arch. Int. Med. **68**: 241, 1941.
26. Baer, D. P., Cecil, R. L., and DuBois, E. F.: Temperature Regulation After Intravenous Injection of Proteose and Typhoid Vaccine, Arch. Int. Med. **29**: 608, 1922.
27. Fiske, C. H.: The Role of Phosphoric Acid in Carbohydrate Metabolism, Proc. Soc. Biol. Chem.—J. Biol. Chem. **41**: 59, 1920.
28. Perlzweig, W. A., Latham, E., and Keefer, C. S.: The Behavior of Inorganic Phosphorus in the Blood and Urine of Normal and Diabetic Subjects During Carbohydrate Metabolism, Proc. Soc. Exper. Biol. & Med. **21**: 33, 1923.
29. Pollack, H., Millet, R. F., Essex, H. E., Mann, F. C., and Ballman, J. L.: Serum Phosphate Changes Induced by Injection of Glucose Into Dogs Under Various Conditions, Am. J. Physiol. **110**: 117, 1934.
30. Free, A. H., and Leonards, J. R.: Serum Phosphorus Changes During the Absorption and Metabolism of Glucose, Galactose, and Xylose, J. Biol. Chem. **149**: 203, 1943.
31. Bollinger, A., and Hartman, F. W.: Observations on Blood Phosphates as Related to Carbohydrate Metabolism, J. Biol. Chem. **64**: 91, 1925.

32. Kerr, S. E., and Blish, M. E.: The Effect of Insulin on Phosphorus Compounds of Muscle, J. Biol. Chem. **97**: 11, 1932.
33. Cori, G. T., and Cori, C. F.: Changes in Hexosephosphate, Glycogen and Lactic Acid During Contraction and Recovery of Mammalian Muscle, J. Biol. Chem. **99**: 493, 1933.
34. Friedlander, K., and Rosenthal, W. G.: Ueber den Einfluss des Phosphorsäureions auf den Blut- und Harnzucker des normalen und des diabetischen Organismus, Arch. f. exper. Path. u. Pharmakol. **112**: 65, 1926.
35. Hench, P. S., and Slocumb, C. H.: Results of Fever Therapy for Gonorrheal Arthritis, Chronic Infectious (Atrophic) Arthritis, and Other Forms of "Rheumatism," Proc. Staff Meet., Mayo Clin. **10**: 202, 1935.
36. Fenn, W. O.: The Role of Potassium in Physiological Processes, Physiol. Rev. **20**: 377, 1940.
37. Zwemer, R. L., Sims, E. A. H., and Coggeshall, L. T.: The Plasma Potassium Level During Malarial Infection in Monkeys and Man, Am. J. Trop. Med. **26**: 687, 1940.
38. Stevenson, Dwight S.: The Use of Calcium Gluconate in the Treatment of Malarial Chills, Puerto Rico J. Pub. Health & Trop. Med. **19**: 602, 1944.
39. Schmidt, C. L. A., and Greenberg, D. M.: Occurrence, Transport, and Regulation of Calcium, Magnesium, and Phosphorus in Animal Organism, Physiol. Rev. **15**: 297, 1935.
40. Shohl, A. T.: Mineral Metabolism—Calcium and Magnesium, Ann. Rev. Biochem. **2**: 207, 1933.
41. Sjollema, B.: Nutritional and Metabolic Disorders in Cattle, Nutrition Abstr. & Rev. **1**: 621, 1932.
42. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Vol. 2, Baltimore, 1932, Williams & Wilkins Company, p. 863.
43. Simpson, W. H.: Influence of Artificial Fever on Chloride Metabolism, J. A. M. A. **100**: 67, 1933.
44. Hanger, F. M.: Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin-Cholesterol Emulsions, J. Clin. Investigation **18**: 261, 1939.
45. MacLaglan, N. F.: Thymol Turbidity Test; a New Indicator of Liver Dysfunction, Brit. J. Exper. Path. **25**: 234, 1944.
46. Mirsky, I. A., von Brecht, R., and Williams, L. D.: Hepatic Dysfunction in Malaria, Science **99**: 20, 1944.
47. Sabin, A. B., Philip, C. B., and Paul, J. R.: Phlebotomus (Sandfly) Fever, J. A. M. A. **125**: 693, 1944.
48. Bronstein, L. H., and Reid, R. D.: The Cephalin-Cholesterol Flocculation Test in Malaria, Proc. Soc. Exper. Biol. & Med. **60**: 140, 1945.
49. Steinberg, A.: Desoxycholic Acid—Cholesterol Flocculation Test and Its Comparison With Other Tests of Liver Dysfunction. In preparation.
50. Danielson, W. H., Steeher, R. M., Muntwyler, E., and Myers, V. C.: The Acid-Base Balance of the Blood Serum in Hyperthermia, Am. J. Physiol. **123**: 550, 1938.

NORMAL SEDIMENTATION RATES IN ACTIVE PULMONARY TUBERCULOSIS

A Study of 1,066 Cases in a Naval Tuberculosis Center

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ERYTHROCYTE sedimentation tests long have been of value in determining the activity of tuberculous lesions. Like many diagnostic aids, however, the test has been abused and misinterpretations occasionally have caused loss of faith[1] in a worth-while procedure. Recent experience in a large naval tuberculosis center (Sampson Naval Hospital) has provided opportunity to observe the use and abuse of sedimentation rates for the diagnosis and evaluation of pulmonary tuberculosis.

Patients admitted to this center offered an unusually good clinical history of the infection. Each man in the Navy carries with him a so-called health record in which is entered full reports of any medical attention tendered him while in service. Thus, each patient brought with him a fairly complete medical history containing reports of previous medical studies, x-rays, and laboratory procedures.

In a rather alarming number it was seen that men with active lesions had been under observation at a recent date because of their pulmonary infiltration. Studies during the previous admissions resulted in the diagnosis of inactive disease with consequent "back to duty" discharge. The men often returned later, however, with more advanced lesions. A study of such records indicates the continuation of a fairly widespread belief that active tuberculosis always will be reflected by an increased sedimentation rate.[2-5]

On the basis of the foregoing considerations, a study was made of 1,066 patients with active pulmonary disease admitted to the Sampson Naval Hospital between January, 1945, and March, 1946. They were all essentially new patients never having been treated previously for tuberculosis except at the activity which forewarded them to the tuberculosis center. In some instances bed rest of varying degrees and duration had preceded their admission, but this did not prevent their inclusion in the study if other findings indicated currently active pulmonary tuberculosis.

The sedimentation rate of each patient was determined within the first few days after admisssion by the Cutler procedure.[6] Normal limits of sedimentation in one hour by this method are 2 to 8 mm. for men and 2 to 10 mm. for women. No correction is necessary for hematocrit values.[3, 7, 8] The Cutler method has been found to compare favorably with other sedimentation procedures.[9, 10]

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From the group of 1,066 cases, patients were selected in whom the diagnosis of active pulmonary tuberculosis was relatively certain. The criteria for this diagnosis were as follows: (1) an active pulmonary lesion by x-ray examination; (2) the presence of tubercle bacilli on three separate occasions in the sputum and/or gastric washings by smear and/or culture; and (3) no evidence of pleural fluid, pneumothorax, thoracoplasty, and no tuberculosis or other disease elsewhere in the body.

There were 300 cases meeting these qualifications. Of these, 61 (or 20.3 per cent) had sedimentation rates within the normal range (Table I).

TABLE I

GROUPS	NUMBER OF CASES	NUMBER OF CASES WITH NORMAL SEDIMENTATION RATE	PER CENT OF CASES WITH NORMAL SEDIMENTATION RATE
I (a) and (b)	*300*	*61*	*20.3*
II (a) and (c)	*240*	*126*	*52.5*
I and II	540	187	34.6
III (a) and (d)	*80*	*42*	*52.5*
I, II, and III	620	229	36.9

(a) Active pulmonary lesion by x-ray examination with no evidence of tuberculosis or other disease elsewhere in the body.

(b) Tubercle bacilli in the sputum and/or gastric washings by smear and/or culture on *three* occasions.

(c) Tubercle bacilli in the sputum and/or gastric washings by smear and/or culture on *one* or *two* occasions.

(d) No positive bacteriologic findings but definite cavitation by x-ray examination.

A second group was then selected in which all the previously mentioned criteria were fulfilled, except that a positive smear and/or culture of sputum and/or gastric washings was found on only one or two occasions. There were 240 cases meeting these qualifications, making a total, with the first group, of 540 cases. Of these, 187 (or 34.6 per cent) had sedimentation rates within the normal range.

Finally, a group of cases was added in which all these criteria were fulfilled, except that no positive bacteriologic findings were recorded. Instead, these patients had definite cavitation as determined by the roentgenologist, mostly on stereoscopic examinations. Cases in which cavitation was only suspected were not included.

The final group of 80, when added to the first two groups, made a total of 620 cases. Of all these, 229 (or 36.9 per cent) had sedimentation rates within the normal range.

The remaining 446 cases were complicated by pleural effusion, pneumothorax, questionable x-ray shadows, or lack of positive bacteriology. No unchallenged conclusions could be drawn from sedimentation rates in this group.

DISCUSSION

The incidence of normal sedimentation rates in active pulmonary tuberculosis in this series is strikingly large and might be difficult to accept if other examiners had not reported comparable results.

Thiele[11] has found that 25 per cent of open tuberculosis, 85 per cent of the cirrhotic type, 68.3 per cent of the cirrhotic-productive type, 42.3 per cent of the productive type, and 47 per cent of the cavernous type of tuberculosis have normal sedimentation rates. Leitner[12] reported normal values in 32.1 per cent, Pinner[13] in 40 per cent, and Bobrowitz[14] 45 per cent normal sedimentation rates in minimal active cases. Overholt and Wilson[15] state categorically that "the vast majority of early lesions reflect no change in sedimentation rates."

Berg[16] has reported eighteen cases, Banyai and Caldwell[17] one hundred and eleven, Tillisch[18] nineteen, and Breuhaus[19] seven instances of normal sedimentation rates with active disease, the last in the face of advancing cavitation. Banyai and Anderson[20] stated that 7.35 per cent of cases with active tuberculosis had normal sedimentation rates, Sopp[21] that 6.6 per cent of dispensary patients, and Rest[22] that nearly one-quarter of demonstrable cases gave similar readings over a long period of time.

Pessar and Hurst[23] reported that 18 per cent of men and 12 per cent of women with active tuberculosis gave lower than expected values. De Cecio and Elwood[8] found that in 224 patients admitted to the center with normal sedimentation rates, 61 per cent had active disease.

Several factors might be considered to explain the relatively high proportion of normal sedimentation rates in patients at the tuberculosis center. Many of these men were found to have roentgenologic evidence of tuberculosis on routine discharge examination and therefore were hospitalized with few or no symptoms of the disease. Also, the short previous hospitalization (average estimated at seven weeks) before admission to the Sampson Naval Hospital may have allowed the secondary inflammatory reactions to subside. Some observers believe that secondary reactions are chiefly responsible for the elevation of the sedimentation rate.

It seems logical to postulate that, as tuberculosis case-finding programs are expanded and more patients are found before the onset of secondary reactions with symptoms, normal sedimentation rates may also be found more frequently.

A study of active cases at the center according to the degree of anatomic involvement (by National Tuberculosis Association standards,[24] is interesting. In the first group of 300 relatively certain cases of active pulmonary disease, 20.9 per cent were classified as minimal, 55 per cent moderately advanced, and 24.1 per cent far advanced. Of the cases with normal sedimentation rates in that group, 44.4 per cent were minimal, 48.2 per cent moderately advanced, and 7.4 per cent far advanced. In this last 7.4 per cent was included one patient with advancing miliary disease who had a repeatedly normal sedimentation rate.

In these three classifications the anatomic involvement was roughly paralleled by the sedimentation rates, as has been found by others including Levinson[25] and Wrenn.[26] The average values of the 300 "relatively certain" cases are plotted according to their classification in Fig. 1. It is obvious, however, from the large proportion of normal sedimentation values in all three groups, that this parallel is not of accurate diagnostic value.

The laboratory procedures in this study were done at the Sampson Naval Hospital by Navy-trained technicians. The demobilization program with its resultant personnel changes was in effect during part of this study. Thus, these results must be considered an evaluation of the sedimentation rates in tuberculosis primarily as found in this hospital.

A few of the bacteriologic studies on these cases have been included from other naval laboratories. Re-evaluation of percentages, excluding studies done elsewhere, gives no essential change in the results.

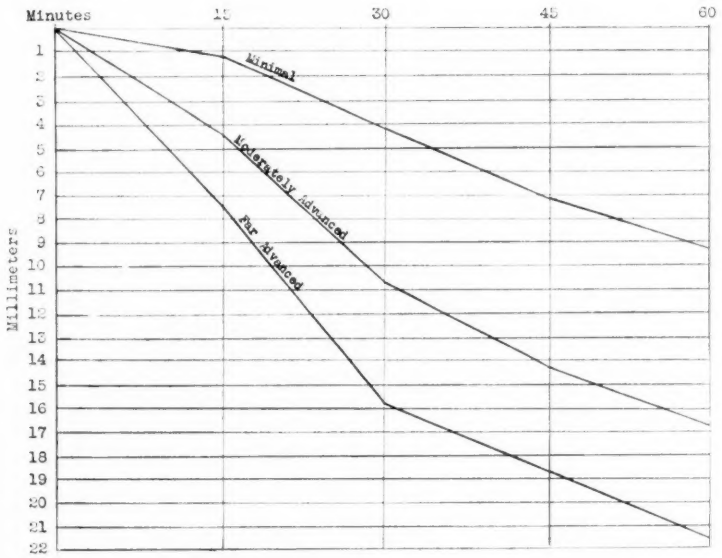

Fig. 1.—Sedimentation rates of 300 "relatively certain" cases of pulmonary tuberculosis averaged according to anatomic involvement.

CONCLUSION

Tuberculosis per se does not always cause an elevated sedimentation rate, according to the conditions and procedure of this study. However, it cannot be denied that the test continues to be of distinct value in analyzing infectious processes, and tuberculosis is an infectious process. The sedimentation rate can continue to be extremely valuable on that basis, but on that basis only.

Except for the pathology and bacteriology of tuberculosis, our entire approach to the disease is indirect. The treatment is indirect and not specific. Diagnosis of the infection is also largely indirect. X-rays, after all, are only shadows which frequently can be misleading and often are misinterpreted and of value to a large extent from a comparative standpoint. We merely use them with these reservations as clearly in mind as possible.

The same can be said for the sedimentation test. It can, and continues to be, of help in tuberculosis until something specific, analogous to agglutination tests for other diseases, can be found. Until that time it must be used sagaciously, with all its limitations in mind.

It has been said[8] of the sedimentation test that it will not make a good physician from a poor one, but it will make a good one better. In tuberculosis, the physician, not the test, must make the diagnosis and determine the status of its pathology.

SUMMARY

1. A study of sedimentation rates in 1,066 cases of active pulmonary tuberculosis is presented.

2. Normal sedimentation rates were found in 20.8, 34.6, or 36.9 per cent of active cases, depending upon the criteria used to determine activity of the disease. This is in agreement with reports from other sources.

3. The sedimentation curve varies roughly, but not uniformly, according to the degree of anatomic involvement by the disease.

4. Tuberculosis per se does not always cause an elevated sedimentation rate. The test, however, continues to be of value in this disease if used with careful regard for its limitations.

REFERENCES

1. Lewis-Fanning, E., and Myers, M.: Prognostic Value of the Blood Sedimentation Rate in Pulmonary Tuberculosis, Brit. M. J. **2**: 125, 1942.
2. Cutler, J. W., and Cohen, L.: The Blood Sedimentation Test as a Routine Procedure in the Tuberculosis Dispensary, Am. Rev. Tuberc. **21**: 347, 1930.
3. Kaminsky, J., and Davidson, D. L.: Observations on the Red Cell Sedimentation in Pulmonary Tuberculosis, Am. Rev. Tuberc. **26**: 282, 1932.
4. Morriss, W. N.: The Value of Erythrocyte Sedimentation Determinations in Pulmonary Tuberculosis, Am. Rev. Tuberc. **10**: 431, 1924.
5. Westergren, A.: On the Stabilitary Reaction of Blood in Pulmonary Tuberculosis, Brit. J. Tuberc. **15**: 72, 1921.
6. Cutler, J. W.: The Graphic Presentation of the Blood Sedimentation Test, Am. J. M. Sc. **171**: 882, 1926.
7. Cutler, J. W., Park, F. R., and Herr, B. S.: The Influence of Anemia on Blood Sedimentation, Am. J. M. Sc. **195**: 734, 1938.
8. De Cecio, T., and Elwood, B. J.: Erythrocyte Sedimentation, Am. Rev. Tuberc. **39**: 748, 1939.
9. Greisheimer, E. M., Treloar, A. E., and Ryan, M.: The Inter-relation of Cutler, Linzenmeier, and Westergren Sedimentation Tests, Am. J. M. Sc. **187**: 213, 1934.
10. Ham, T. H., and Curtis, F. C.: Sedimentation Rates of Erythrocytes, Medicine **17**: 447, 1938.
11. Thiele, G.: Ueber den Wert der Bestimmung der Blutkörperchensenkungsgeschwindigkeit für die Diagnosen- und Prognosenstellung bei der Lungentuberkulose, Beitr. z. Klin. d. Tuberk. **85**: 302, 1934.
12. Leitner, J.: Die Bedeutung des weissen Blutbildes im Verhältnis zur Blutkörperchensenkungsgeschwindigkeit bei der klinischen Beurteilung einzelner Formen der Lungentuberkulose, Beitr. z. Klin. d. Tuberk. **86**: 297, 1935.
13. Pinner, M.: Pulmonary Tuberculosis in the Adult, Springfield, Ill., 1945, Charles C Thomas, Publisher.
14. Bobrowitz, I. D., and Dwork, R. E.: The Early Diagnosis of Minimal Pulmonary Tuberculosis, New England J. Med. **234**: 10, 1946.
15. Overholt, R. H., and Wilson, N. J.: Silent and Masquerading Intrathoracic Lesions, New England J. Med. **234**: 169, 1946.
16. Berg, S.: Offene Lungentuberkulose mit niedrigem bzw. normalem Blutsenkungswert, ihr Vorkommen, ihre Prognose und Therapie, Beitr. z. Klin. d. Tuberk. **83**: 551, 1933.
17. Banyai, A. L., and Caldwell, E.: Normal Sedimentation Rates in Open Pulmonary Tuberculosis, Am. Rev. Tuberc. **38**: 491, 1938.
18. Tillisch, A.: Quoted in Berg, S.[16]
19. Breuhaus, R.: Sedimentation Test in Tuberculosis, Med. Welt. **9**: 1867, 1935.

20. Banyai, A. L., and Anderson, S. V.: Erythrocyte Sedimentation Test in Tuberculosis, Arch. Int. Med. **46**: 787, 1930.
21. Sopp, J. W.: Die Bedeutung der Bestimmung der Blutkörperchensenkungsgeschwindigkeit in der Tuberkulosefürsorge, Beitr. z. Klin. d. Tuberk. **87**: 86, 1935.
22. Rest, A.: The Erythrocyte Sedimentation Rate in Pulmonary Tuberculosis, Tubercle **19**: 307, 1938.
23. Pessar, H. T., and Hurst, A.: Erythrocyte Sedimentation, Am. Rev. Tuberc. **38**: 495, 1938.
24. Diagnostic Standards and Classifications of Tuberculosis, Tr. Nat. Tuberc. A., New York, 1940, 32 pp.
25. Levinson, S. A.: The Suspension Stability Rate of Erythrocytes in Pulmonary Tuberculosis, Am. Rev. Tuberc. **7**: 264, 1923.
26. Wrenn, H. T.: The Blood Sedimentation Test in Pulmonary Tuberculosis, U. S. Vet. Bur. M. Bull. **3**: 703, 1927.

EFFECT OF 3,3′ METHYLENEBIS (4 HYDROXYCOUMARIN) ON VITAMIN A DEFICIENT DUCKS

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THE experimental observations of Wakin and associates[1] indicate that dicumarol* in large doses may have a toxic action on certain animals prior to the onset of hypoprothrombinemia. Several studies have shown that hypoprothrombinemia resulting from dicumarol requires a latent period of approximately twenty-four hours.[2-4] Some dogs given very large doses of this drug become dyspneic, have convulsions, and are comatose preceding death. No hemorrhagic manifestations occurred during life nor were hemorrhages found at autopsy in these dogs.[5] Dogs given intravenously 40 to 60 mg. of dicumarol per kilogram of body weight usually died within an hour without showing any changes in either the prothrombin or the coagulation times.[1]

Richards and Cortell[6] in 1942 observed that guinea pigs depleted of vitamin C succumb earlier to the effects of dicumarol. They said that "a connection between vitamin C level and sensitivity to dicumarol must be considered possible." Persons dying from infections may have a low vitamin A content in the liver.[7-9] Apparently the mechanism of lowered resistance to infection in vitamin A deficiency is not well understood. Susceptibility may not be directly related to the deficiency, since some individuals show a normal amount of vitamin A in the presence of disease. Bessey and Wolback[10] have called attention to the fact that "during a prolonged deficiency, the reserve (of vitamin A) is gradually depleted, normal cell functions are suspended, and pathologic change develops."

During recent studies on malaria in ducks it was observed that normal ducklings frequently died within three or four hours following the oral administration of large doses of dicumarol. There was observed also a variation in the time of death following the oral administration of dicumarol in ducks fed a vitamin A deficient ration when compared with birds fed a standard commercial ration. These experimental observations are reported at this time.

METHODS AND MATERIAL

Ducks used in these studies were white Pekins obtained when 1 day of age from a commercial hatchery. They were kept in small batteries and given food and water ad lib. The standard ration was duck Startena and duck Growena. The composition of the low vitamin A ration and the standard rations are as follows:

From the Department of Pathology, University of Arkansas School of Medicine.

Aided by a grant from the John and Mary R. Markle Foundation and Eli Lilly and Company, Indianapolis, Ind.

The dicumarol was supplied by The Wm. S. Merrell Company, Cincinnati, Ohio.

The vitamin A deficiency ration was supplied by the Ralston Purina Mills, St. Louis, Mo., through the courtesy of Dr. Homer Patrick.

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*Dicumarol is the collective trade-mark of the Wisconsin Alumni Research Foundation and is used for 3,3′ methylenebis (4 hydroxycoumarin).

LOW VITAMIN A DUCK RATION

Mineralized salt	0.25
Puriflave (250)	1.00
Calcium carbonate	2.00
Soybean oil meal	18.00
Wheat germ	5.00
Fish meal (60%)	6.00
Meat scraps	4.00
Brewers' yeast	5.00
Ground wheat	19.65
White corn	29.00
Ground oats	5.00
Molasses	5.00
Dry D (2,000 D)	0.1

Vitamin mixture (milligram per 100 Gm. ration)

Thiamin	0.5
Riboflavin	0.5
Choline	200.0
Folic acid	0.5
Niacin	5.0

Ration I prepared Jan. 2, 1946; II, Jan. 28, 1946; and III, March 10, 1946.

The following data are all that are available on the two commercial rations:

DUCK STARTENA RATION

Crude protein not less than	20.0%
Crude fat not less than	3.5%
Crude fiber not more than	6.5%
Nitrogen-free extract not less than	44.0%

Ingredients: Corn meal, ground oats, wheat bran, alfalfa leaf meal, meat scrap, fish meal, soybean oil meal, liver meal, corn gluten meal, wheat standard middlings, wheat germ, riboflavin supplement, vitamin A and D feeding oils, D activated animal sterol, 2% calcium carbonate (limestone), .25% iodized salt, .01% manganese sulfate.

DUCK GROWENA RATION

Crude protein not less than	18.0%
Crude fat not less than	3.5%
Crude fiber not more than	6.0%
Nitrogen-free extract not less than	48.0%

Ingredients: Meat scrap, soybean oil meal, corn gluten meal, fish meal, liver meal, ground oats, corn meal, wheat bran, wheat gray middlings, wheat germ, riboflavin supplement, vitamin A feeding oil, D activated animal sterol, 2% calcium carbonate (limestone), .25% iodized salt, .01% manganese sulfate.

Vitamin A (alphalin*) was used to supplement these rations. The contents of one capsule (25,000 units) was dropped into the mouth the younger birds and the entire capsule was given by mouth to ducks over 10 days of age. The vitamin A was given at intervals of one to three days during the time of the experiment.

Ducks were selected from the groups at different intervals and their liver was removed immediately following decapitation. The tissue was kept frozen until the vitamin A content was determined by the antimony trichloride reaction.†

The dicumarol was given by mouth. The quantity varied; in each experiment, however, a group of ducks from one hatching was used and an equal-sized dose of dicumarol was given to each bird. The amount is recorded in each experiment.

The ducks were carefully observed during the time of the experiment for the appearance of clinical manifestations. The number that died and the time of death were recorded. The average weight of the ducks used in each experiment is given in Table I.

*Eli Lilly's preparation of vitamin A.

†These tests were made by Dr. Homer Patrick of the Ralston Purina Mills, St. Louis, Mo.

TABLE I. LETHAL EFFECT OF DICUMAROL ON VITAMIN A DEFICIENT DUCKS

EXPERIMENT	NUMBER OF DUCKS IN GROUP	AGE IN DAYS	AVERAGE WT. OF GROUP (GM.)	RATION	EXPERIMENTAL DAYS—AMOUNT OF DICUMAROL 0	1	2	3	4	5	6
A	5	15	360	Vitamin A deficiency Ration II*	50†	75	100	50			
					2‡	3	4	5			
	5	15	374	Standard ration	50	75	100	50			
					0	0	0	0			
B	2	17	630	Vitamin A deficiency Ration II	100	50					
					1	1	1				
	5	17	477	Standard ration	100	50					
					0	0	0				
C	12	17	469	Vitamin A deficiency Ration III	50	50	100	200	200		
					0	0	2	5	10	10	10
	10	17	489	Standard ration	50	50	100	200	200		
					0	0	1	1	8	9	9
D	12	17	469	Vitamin A deficiency Ration III	100	100	150	200	200		
					5	5	5	6	11	12	12
	10	17	481	Standard ration	100	100	150	200	200		
					1	1	1	3	7	7	7
E	8	18	570	Standard ration Plus vitamin A	100	150	150	150	150		
					1	2	2	2	2	2	2
	10	18	500	Standard ration	100	150	150	150	150		
					0	5	5	6	8	8	8
F	9	18	563	Standard ration Plus vitamin A	150	150	150	150	150		
					3	3	3	3	3	3	4
	10	18	504	Standard ration	150	150	150	150	150		
					8	8	8	8	8	8	8
G	4	28	1,065	Vitamin A deficiency Ration I	250	300					
					0	3	3				
	4	28	1,121	Vitamin A deficiency Ration I plus vitamin A	250	300					
					0	0	0				
H	12	18	496	Vitamin A deficiency Ration III	100	100	100	150	150		
					1	1	2	5	8	8	
	12	18	500	Vitamin A deficiency Ration III plus vitamin A	100	100	100	150	150		
					1	1	1	1	10	10	
	12	18	584	Standard ration	100	100	100	150	150		
					0	0	0	5	5	6	8
I	3	40	1,476	Vitamin A deficiency Rations I and II	300	300					
					0	1	2				
	3	40	1,693	Vitamin A deficiency Rations I and II plus vitamin A	300	300					
					1	1	1				
	1	40	1,685	Standard ration	300	300					
					0	0	0				

*Composition of different rations is given in body of text.
†Milligrams of dicumarol given each day is shown as the upper figure.
‡Total number of deaths is shown as the lower figure.

EXPERIMENTAL EFFECT OF DICUMAROL ON DUCKS FED A VITAMIN A DEFICIENT RATION

A total of 149 ducks, as shown in Table I, were used in these experiments. In Experiment A, ten ducks were used, five of which were fed the vitamin A deficient ration II and five the standard rations. When 15 days of age these birds were given daily 50, 75, 100, and 50 mg. of dicumarol. On the third day each of the five birds fed the vitamin A deficient ration was dead; the five fed the standard rations had survived.

In Experiment B, seven ducks were used, two of which were fed the vitamin A deficient ration II and five the standard ration. When 17 days of age these ducks were given daily 100 and 50 mg. of dicumarol. On the second day one of the two ducks fed the vitamin A deficient ration was dead; the five fed the standard rations had survived.

In Experiment C, twenty-two ducks were used, twelve of which were fed the vitamin A deficient ration III and ten the standard rations. When 17 days of age these ducks were given daily 50, 50, 100, 200, and 200 mg. of dicumarol. On the third day five from the group of twelve fed the vitamin A deficient ration were dead, while only one from the group of ten fed the standard rations had died. On the sixth day ten ducks from the group of twelve fed the deficient ration were dead; nine were dead from the group of ten fed the standard rations.

In Experiment D, twenty-two ducks were used, twelve of which were fed the vitamin A deficient ration III and ten the standard rations. When seventeen days of age these birds were given daily 100, 100, 150, 200, and 200 mg. of dicumarol. Six hours following the first dose of dicumarol five ducks from the group of twelve fed the deficient ration were dead while only one had died in the group fed the standard rations. On the fourth day eleven of the twelve ducks fed the deficient ration had died, while seven were dead from the group of twelve fed the standard rations.

In these four experiments, seventeen ducks (54.8 per cent) were dead by the third day from the group of thirty-one fed the vitamin A deficient rations. Only four (13.3 per cent) were dead from the group of thirty ducks fed the standard rations for an equal period.

EFFECT OF DICUMAROL ON DUCKS GIVEN A HIGH VITAMIN A RATION

In this experiment, forty-five ducks were used.

In Experiment E, eight ducks were fed the standard rations supplemented with vitamin A, while ten ducks were fed only the standard rations. On the eighteenth day these birds were given daily 100, 150, 150, 150, and 150 mg. of dicumarol. Six hours following the second dose of dicumarol five of the ten ducks fed the standard rations had died, while only two from the group of eight fed the standard rations supplemented with vitamin A were dead.

In Experiment F, nineteen ducks were used, ten of which were fed the standard rations, while nine were fed the standard rations supplemented with vitamin A. On the eighteenth day of the experiment these ducks were given daily 150 mg. of dicumarol for five days. Six hours after the first dose of dicumarol was given six of the ten ducks on the standard rations had died, while only three from the group of nine on the standard rations supplemented with vitamin A were dead.

In Experiment G, eight ducks were used, four of which were fed the vitamin A deficient ration I and four the same deficient A ration supplemented with vitamin A. On the twenty-eighth day of the experiment these birds were given daily 250 and 300 mg. of dicumarol. On the second day three of the

four ducks fed the standard ration were dead; neither of the four birds fed the vitamin A deficient ration supplemented with vitamin A died.

Experiments E and F show that ducks given large amounts of vitamin A are more resistant to the acute action of dicumarol than birds of similar age fed the standard rations. In the former group seventeen ducks were used and in the latter twenty. On the third day five ducks (29.4 per cent) had died from the group fed the standard rations supplemented with vitamin A, while fourteen (70 per cent) were dead from the group fed only the standard rations.

EFFECT OF DICUMAROL ON DUCKS GIVEN A LARGE, MEDIUM, AND LOW QUANTITY OF VITAMIN A

In these experiments a total of forty-three ducks were used. To obtain ducks with the smallest amount of vitamin A, the vitamin A deficient rations were fed. The standard rations were fed to obtained birds with a medium quantity of vitamin A, while those with the largest quantity of vitamin A were fed the vitamin A deficient ration III supplemented with large amounts of vitamin A.

In Experiment H, thirty-six ducks were used. Three groups of twelve birds each were given one of the three rations as previously stated to obtain the three different levels of vitamin A. On the eighteenth day these ducks were given daily 100, 100, 100, 150 and 150 mg. of dicumarol. On the third day five ducks had died from the group fed the vitamin A deficient ration, five were dead from the group fed the standard rations, while only one had died from the group of twelve ducks fed the vitamin A deficient ration supplemented with vitamin A.

In Experiment I, seven ducks were used. Three of these ducks were fed the vitamin A deficient rations I and II, three were fed the vitamin A deficient rations supplemented with vitamin A, and one duck was fed the standard rations. On the fortieth day of the experiment the seven ducks were given 300 mg. of dicumarol daily for two doses. On the second day two of the three ducks fed the vitamin A deficient rations were dead; one of the three birds given the vitamin A deficient ration supplemented with vitamin A was dead: and the one bird fed the standard rations during this time survived the effects of the dicumarol.

In these two experiments fifteen ducks were fed the vitamin A deficient rations and given dicumarol; from this group seven (46.6 per cent) died. Fifteen ducks were fed the vitamin A deficient ration supplemented with vitamin A and two (13.3 per cent) died. Thirteen ducks were fed the standard rations and five (38.4 per cent) were dead by the third day.

The respiratory rate was greatly increased within two or three hours after the larger doses of dicumarol were given. This increase in the respiratory rate persisted for two or three hours during which time many of the ducks died. The ducks that survived the first large dose of dicumarol showed less severe clinical reaction on subsequent days to the same amount of dicumarol. The ducks that died appeared to be in satisfactory condition until a few minutes before death except for their rapid respiration. During the last few minutes

of life these dicumarol-treated ducks usually had one or more convulsions. Hemorrhages usually were absent in these ducks that succumb after the second and third dose of dicumarol. Some bleeding was present, however, in the tissues of the ducks that died on the fifth and sixth days of the experiment.

VITAMIN A CONTENT OF THE LIVER OF DUCKS

Samples from the groups of ducks were taken to determine the amount of vitamin A present in the liver. On the eighteenth day the liver from two ducks fed the vitamin A deficient ration I showed 1.5 and 14.4 U.S.P. units per gram of liver. This same deficient ration was given two other ducks and vitamin A was used to supplement it. The liver from these two ducks showed, respectively, 1,360 and 1,520 U.S.P. units per gram of liver. Two ducks were fed the vitamin A ration III and on the eighteenth day the liver from each was removed. Neither of these two birds had any vitamin A in the liver. Two ducks fed the standard rations for eighteen days had, respectively, 13.8 and 18.2 U.S.P. units of vitamin A per gram of liver tissue. The vitamin A deficient ration III was supplemented with vitamin A and fed to two ducks for eighteen days. One duck had 582 and the second 546 U.S.P. units of vitamin A in the liver.

WEIGHT OF VITAMIN A DEFICIENT DUCKS

The following is the average weight of groups of ducks fed the vitamin A deficient ration III, vitamin A deficient ration III supplemented with vitamin A, and the standard rations:

DUCKS	AGE IN DAYS	RATION	AVERAGE WEIGHT (GM.)
24	15	Vitamin A deficient ration	425
20	15	Standard rations	442
12	18	Vitamin A deficient ration	496
12	18	Vitamin A deficient ration plus vitamin A	500
12	18	Standard rations	584
28	20	Vitamin A deficient ration	623
20	20	Vitamin A deficient ration plus vitamin A	516

Ducks fed the vitamin A deficient rations usually were slightly lighter than those kept on the standard rations. The variation in weight of the different groups, however, was not very great.

DISCUSSION

The observation that ducks fed large amounts of vitamin A are relatively resistant to large doses of dicumarol is interesting since vitamin A is stored in the liver and dicumarol has been found to produce necrosis in the liver of laboratory animals.[1, 6] Apparently the mechanism has not been established for the development of hepatic necrosis following large doses of dicumarol. This action occurs, however, before the hypoprothrombinemia.[1] No specific pathologic changes have been observed in the liver of ducks given large doses of dicumarol for as long as five days. The cause of the acute death in these ducks is not known. It has been observed, however, that a 50 per cent decrease

may occur in the number of red blood cells within two to four hours following the oral administration of large doses of dicumarol. The plasma at this time does not show any hemolysis; the decrease in the hematocrit reading seems to parallel the decrease in the number of red cells. The carbon dioxide content of the plasma is approximately the same as that of normal birds. There is a decrease, however, in the carbon dioxide-combining power of the plasma of dicumarol-treated birds.[12] Experimental observations in our laboratory show that ducks fed the vitamin A deficient ration show no significant variations from the normal in the carbon dioxide content and the combining power of their plasma.

Since the reserve of vitamin A in the liver is low in certain hepatic diseases, it may be advisable to be cautious in the use of dicumarol in the treatment of such individuals if the experimental results as observed in this study should be duplicated in man. Organic and functional changes have been observed in the liver associated with deficiencies other than vitamin A. In chronic vitamin B complex deficiencies functional and fatty changes have been described.[13, 14] A decrease occurs in hepatic function when dogs are fed a black tongue diet.[15] The recent observations of Brunschwig and associates[16, 17] are interesting in that they have shown that both thioglycollic and glycollic acids afford a degree of protection against hepatic cell necrosis due to dietary deficiency and to carbon tetrachloride in the rat. "The results with thioglycollic and glycollic acids are of interest since by their use it would appear that the lipotropic factor and necrosis protecting factor are shown not to be necessarily identical."[17]

It is obvious that the amounts of dicumarol used in these experiments are greatly in excess of those used therapeutically in man; in preliminary experiments, however, it was ascertained that doses of this magnitude were required to obtain the results described. There may be no relation between these experimental studies and the reactions which occur in man. However, the observation that vitamin A deficient ducks are more susceptible to the lethal effect of dicumarol seems of scientific interest.

SUMMARY

The susceptibility of ducks to the acute lethal effect of dicumarol is influenced by the intake of vitamin A. Birds fed a ration supplemented with vitamin A are more resistant to the acute lethal effects of dicumarol than birds fed a ration low in vitamin A. The mechanism by which this effect occurs is not known.

REFERENCES

1. Wakin, K. G., Chen, K. K., and Gatch, W. D.: The Immediate Effects of 3,3′-Methylenebis (4 Hydroxycoumarin) on Experimental Animals, Surg., Gynec. & Obst. 76: 323, 1943.
2. Barker, N. W., Butt, H. R., Allen, E. V., and Bollman, J. L.: The Effect of 3,3′-Methylenebis (4 Hydroxycoumarin) on Blood Coagulation Factors, J. A. M. A. 118: 1003, 1942.
3. Stats, D., and Bullowa, J. G. M.: Effect of a Single Dose of 3,3′-Methylenebis (4 Hydroxycoumarin) Upon Blood Coagulation in Humans, Proc. Soc. Exper. Biol. & Med. 50: 66, 1942.

4. Butt, H. R., Allen, E. V., and Bollman, J. L.: A Preparation From Spoiled Sweet Clover (3,3′-Methylenebis-Bis-3 (4 Hydroxycoumarin) Which Prolongs Coagulation and Prothrombin Time of Blood; Preliminary Report of Experimental and Clinical Studies, Proc. Staff Meet., Mayo Clin. **16:** 388, 1941.
5. Bingham, J. B., Meyer, O. O., and Poble, F. J.: Studies on the Hemorrhagic Agent 3,3′-Methylenebis (4-Hydroxycoumarin) Am. J. M. Sc. **202:** 563, 1941.
6. Richards, R. K., and Cortell, R.: Studies on the Anticoagulant 3,3′-Methylene-Bis-(4 Hydroxycoumarin) Proc. Soc. Exper. Biol. & Med. **50:** 237, 1942.
7. Wolff, L. K.: On the Quantity of Vitamin A Present in the Human Liver, Lancet **223:** 617, 1932.
8. Moore, T.: Vitamin A Reserve of the Human Liver in Health and Disease, Lancet **223:** 669, 1932.
9. Moore, T.: Vitamin A and Carotene; the Vitamin A Reserve of the Adult Human Being in Health and Disease, J. Biol. Chem. **31:** 155, 1937.
10. Bessey, O. A., and Wolback, S.: The Vitamin, American Medical Association, Chicago, 1939, p. 29.
11. Altschule, M. D.: Vitamin A Deficiency, in Spite of Adequate Diet in Congenital Atresia of Bile Ducts and Jaundice, Arch. Path. **20:** 845, 1935.
12. Rigdon, R. H., and McCain, B.: Effect of 3,3′-Methylenebis (4 Hydroxycoumarin) Dicumarol on P. lophurae Infection in Ducks. To be published.
13. Drill, V. A., and Loomis, T. A.: Functional and Fatty Changes in Liver During Chronic Vitamin B Complex Deficiency, Proc. Soc. Exper. Biol. & Med. **61:** 374, 1946.
14. Drill, V. A., Shaffer, C. B., and Leathem, J. H.: Hepatic Injury in Dogs Resulting From a Vitamin B Complex-Free Diet, Proc. Soc. Exper. Biol. & Med. **52:** 328, 1943.
15. Rhoads, C. P., and Miller, D. K.: Hepatic Dysfunction in Dogs Fed Diet Causative of Black Tongue, J. Exper. Med. **67:** 463, 1938.
16. Green, J., and Brunschwig, A.: Protective Action of Certain Compounds Against Dietary Hepatic Drainage in the Rat, Proc. Soc. Exper. Biol. & Med. **61:** 348, 1944.
17. Brunschwig, A., Johnston, C., and Nichols, S.: Carbon Tetrachloride Injury of the Liver; the Protective Action of Certain Compounds, Proc. Soc. Exper. Biol. & Med. **60:** 388, 1945.

SOME FACTORS AFFECTING THE SUSCEPTIBILITY OF RATS TO VARIOUS BARBITURATES

The Effect of Age and Sex

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THE evidence concerning the susceptibility of rats to pentobarbital sodium in regard to age and sex is controversial. Fitch and Tatum,[1] using a dose of 75 mg. per kilogram as the $L.D._{50}$ for rats when injected intraperitoneally, concluded that there is no appreciable difference in the susceptibility of the females and males. But Moir[2] found that the anesthetic responses varied with sex and age as growth proceeded: at first, a greater resistance in the female was seen; later, this was followed by an equality in the response of the two sexes; finally, in the older rats, the male was consistently more resistant. Nicholas and Barron[3] also pointed out that adult females subjected to sodium amytal were affected more than males; in addition they observed that immature rats require a lower dose of this drug to produce anesthesia than do adult rats. In agreement with these workers, Etsten and associates[4] reported that newborn rats are more susceptible to pentobarbital than are adults, while Carmichael[5] noted that the median lethal dose of pentobarbital is less for old rats weighing 150 to 325 grams than for young animals weighing 25 to 260 grams. In the present investigation an attempt was made to re-examine this question with the hope of resolving these contradictions.

METHODS

Three different strains of rats were employed: one from our own colony (C), another from that of the Department of Anatomy (A), and a third from the Rockland Company (R). Unless otherwise indicated in the tables in this paper, rats from our colony were used. The females were nonpregnant. All rats were kept on adequate diets, but food was withheld fifteen or sixteen hours before injecting the drugs, though water was always available.* All barbiturate solutions were freshly prepared and were never older than three hours. The observations were made at 29° Centigrade. The sex, age, weight, strain of rats, rates of injection, and concentration of solutions are presented in the tables.

The criteria used to estimate whether the anesthesia was deep or light were the ear reflex and the posture of the animal. For the ear reflex the response of the pinna is tested with a feather cut to a fine point and inserted into the ear canal. The postural reaction is indicated by the side position, when the rat cannot assume the normal posture due to loss of the righting reflex. The

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*The newborn rats were not fasted. Immediately before injection they were taken away from their mothers. To prevent leakage after intraperitoneal injection, a sphincter clamp was placed on the point of injection before the needle was withdrawn. This clamp was taken off after five minutes. The great majority of the animals which died did so in ten to thirty minutes. Few deaths occurred after three hours with the doses used, and the newborn were returned to their mothers after the three-hour period.

absence of the ear reflex occurs only during deep surgical anesthesia. The interval after the ear reflex has returned, but before the righting reflex is restored, measures the period of light anesthesia.

RESULTS AND DISCUSSION

It is shown in Table I that rats weighing 50 to 100 grams each (four to six weeks old) yield results which vary only within the error of the method in regard to the influence of sex. In the weight range of 101 to 200 grams, however, a sharp increase is observed in the duration of sleep in the females, an increase which is pronounced even at the low dose of 50 mg. per kilogram and which is still more marked with higher doses (Fig. 1). At 90 mg. per kilogram, nine of twenty-nine females died, while only

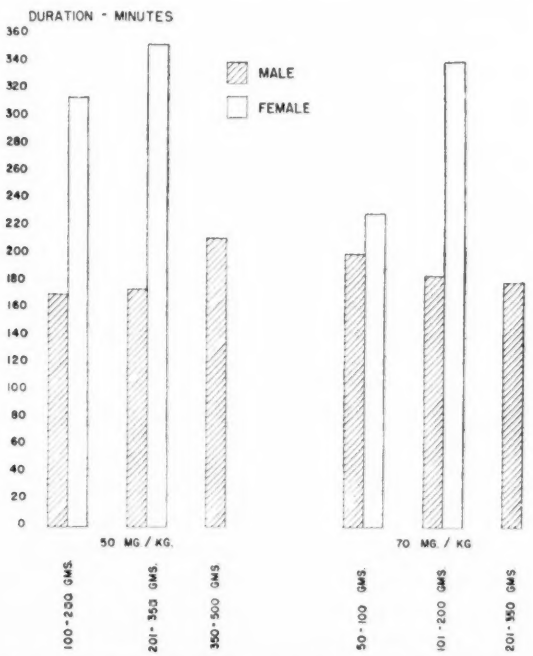

Fig. 1.—Effect of sex on duration of anesthesia. Comparative durations of side position, that is, total period of anesthesia, are presented diagrammatically. Female rats remain anesthesized for a longer period than male animals, after receiving an intraperitoneal injection of pentobarbital (1 per cent solution).

two of twenty-seven males succumbed. In the older group of rats weighing 201 to 350 grams, deaths occur even at 50 mg. per kilogram. At this dose, eleven of forty-eight males and sixteen of thirty females died. In every instance the duration of the narcosis is longer in the female than in the male. When 70 mg. per kilogram was administered to nineteen females in this weight group, all of them died (Fig. 2). Finally, although injection of the oldest males (351 to 500 grams) with 50 mg. per kilogram of pentobarbital resulted in a somewhat lower incidence of mortality (thirteen of sixty-three died) than in the group of males weighing from 201 to 350 grams, the duration of anesthesia was increased

TABLE I. EFFECT OF INTRAPERITONEAL PENTOBARBITAL ON RATS (1 PER CENT SOLUTION)

SEX	WEIGHT RANGE (GRAMS)	40 MG./KG.*		50 MG./KG.		70 MG./KG.		90 MG./KG.		120 GM./KG.	
		MORTALITY†	SIDE POSITION (MIN.)	MORTALITY	SIDE POSITION (MIN.)	MORTALITY	SIDE POSITION (MIN.)	MORTALITY	SIDE POSITION (MIN.)	MORTALITY	SIDE POSITION (MIN.)
M	50 to 100					0/19	200 (166 to 258)	5/39	275 (161 to 406)	13/19	467 (399 to 505)
F						0/16	229 (158 to 334)	3/37	310 (190 to 471)	19/19	
M	101 to 200			0/20	171 (161 to 202)	0/20	184 (124 to 266)	2/27	252 (178 to 388)	1/3	
F				0/16	314 (164 to 466)	0/20	339 (260 to 466)	9/29	391 (280 to 465)		
M	201 to 350	0/15	118 (81 to 133)	11/48	175 (199 to 306)	4/30	179 (138 to 296)	9/24	258 (172 to 375)	15/31	286 (200 to 344)
F				16/30	353 (280 to 420)	19/19					
M	351 to 500			13/63	212 (120 to 365)						
F											

*Dose of pentobarbital administered.

†Each fraction represents the incidence of mortality; the numerator indicates the number of animals that died and the denominator the total number of animals used.

by 22 per cent. It would have been desirable to inject more male rats in the weight range of 201 to 350 grams, but they were not plentiful in our colony. Fitch and Tatum[1] give 75 mg. per kilogram as the $L.D._{50}$ for pentobarbital. Because the age and sex of these rats were not stated, we may point to Table I to show that this figure may be derived by employing both male and female rats weighing from 201 to 350 grams.

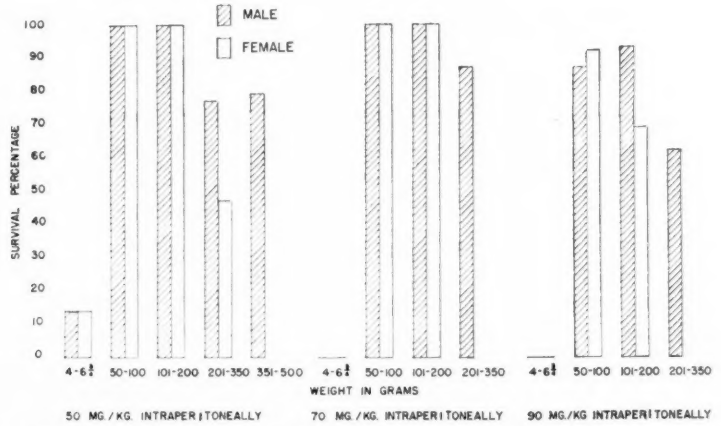

Fig. 2.—Effect of weight and sex on resistance to pentobarbital. Presented in diagrammatic form is the survival percentage, that is, resistance of male and female rats to three different doses of pentobarbital, namely, 50, 70, and 90 mg. per kilogram. The largest of these three doses shows most clearly that the resistance is lowest in the newborn, reaches a maximum sometime while the weight changes from 50 to 100 grams, and then recedes. But even with the lower doses the increasing susceptibility of the aged rats is seen.

Moir[2] observes that in male and female rats weighing about 45 grams each there is a slightly greater resistance in the females. This difference becomes marked when the female rats weigh 55 grams and the male rats 59 grams. This stage of greater female resistance is followed by another in which there is no appreciable difference between the two sexes. This period in which both sexes respond in the same way takes place while the female rats grow from 63 to 81 grams and the male from 68 to 85 grams. After that the relation between the male and female is the opposite of the earlier reaction, for the males become more resistant. The comparatively diminished female resistance is even further impaired when the females weigh 104 grams and the males 113 grams. Thus, the similarity between the reactions of the male and female rats weighing from 50 to 100 grams may be explained by Moir's work as including three periods: (1) greater female resistance, (2) no difference between sexes, and (3) greater male resistance.

It is shown in Table II that a difference in susceptibility is observed even among newborn rats if they are grouped according to weight. The larger and more vigorous newborn rats can tolerate a greater amount of the drug. Yet, with doses of 70 mg. per kilogram or more (results not included in Table II) the mortality is 100 per cent, even with the heaviest members of the newborn group. Irrespective of the differences among the newborn as a group, they are more sensitive to barbiturates than are the adults.

TABLE II. EFFECT OF INTRAPERITONEAL PENTOBARBITAL ON RATS LESS THAN TWENTY-FOUR HOURS OLD

WEIGHT RANGE (GRAMS)	DOSE (30 MG./KG.)	DOSE (40 MG./KG.)	DOSE (50 MG./KG.)
4 to 4¾	5/7*	6/7	1/1
5 to 5¾	6/10	19/27	7/7
6 to 6¾	3/19	4/24	4/6

In order to maintain the same small volume of injection, the concentrations were varied: 30 mg. per kilogram, 0.15 per cent; 40 mg. per kilogram, 0.20 per cent; and 50 mg. per kilogram, 0.25 per cent.

*Each fraction represents the incidence of mortality; the numerator of fraction indicates the number of animals that died and the denominator the number of animals used.

It is generally accepted that a reciprocal relation exists between metabolic rate and narcotic effect. In the present investigation it has been possible to push this analysis one step further, and it is suggested that this relationship rests for the most part on the metabolic rate of the brain rather than that of the entire body. It is true that in rats the form of the curves for basal metabolic rate[6] and cerebral metabolic rate[7] are similar on a basis of age. Both curves fluctuate within their lowest levels for approximately the first three weeks of life before rising rapidly to a maximum at the end of the seventh week and then receding very slowly toward old age. However, the early changes of basal metabolism rate would seem to depend on the entire organism rather than on the metabolism of any organ, for the metabolism of kidney and liver slices from newborn rats is found to be approximately the same as in the mature animal.[8] Cerebral metabolism, on the contrary, is much lower in the newborn than in the adult,[7] an observation which may explain the lessened resistance of the neonate to barbiturate. This relationship between cerebral metabolism rate and narcosis is to be expected since the primary effect of narcosis is exerted on the brain.

Tables III and IV represent a comparative study of pentothal and compound 897 (N-methyl-pentobarbital).* With strain A, but not with the other strains, the intravenous M.L.D. for a male rat weighing 360 to 460 grams is 50 mg. per kilogram for pentothal and 40 mg. per kilogram for compound 897.

TABLE III. INTRAVENOUS INJECTION OF PENTOTHAL (1 PER CENT SOLUTION)

STRAIN*	SEX	WEIGHT RANGE (GRAMS)	INJECTION TIME IN MINUTES	DOSE MG./KG.	MORTALITY†	EAR REFLEX ABSENT (MINUTES)		SIDE POSITION MAINTAINED (MINUTES)	
						AVERAGE	RANGE	AVERAGE	RANGE
R	M	300 to 420	1½	60	3/5	250	141 to 360	375	290 to 460
A	M	360 to 460	1½	50	4/7	178	403 to 540	454	403 to 540
R	M	300 to 420	1½	50	0/5	127	28 to 360	329	303 to 500
C	M	230 to 250	1½	50	0/3	37	32 to 39	254	128 to 420
C	M	260 to 310	½	30	0/9	16	8 to 28	32	16 to 81
C	F	160 to 200	½	30	0/9	15	8 to 20	26	15 to 40
R	M	300 to 420	1½	25	0/7	19	8 to 30	46	19 to 82
R	M	300 to 420	½	12.5	0/7	+	+	8½	4 to 11
C	M	114 to 150	½	12.5	0/7	+	+	Not in side position	

*Strain R came from the Rockland Company; Strain A is that maintained by the Department of Anatomy, Albany Medical College; Strain C came from our own colony in the Department of Physiology and Pharmacology.

†Each fraction represents the incidence of mortality; the numerator indicates the number of animals that died and the denominator the total number of animals used.

*We are grateful to Dr. R. K. Richards of the Abbott Laboratories, North Chicago, Ill., for supplying this drug.

TABLE IV. INTRAVENOUS INJECTION OF COMPOUND 897 (1 PER CENT SOLUTION)

STRAIN*	SEX	WEIGHT RANGE (GRAMS)	INJECTION TIME IN MINUTES	DOSE MG./KG.	MORTALITY†	EAR REFLEX ABSENT (MINUTES)		SIDE POSITION MAINTAINED (MINUTES)	
						AVERAGE	RANGE	AVERAGE	RANGE
R	M	300 to 420	1½	50	4/5	62	---	120	---
A	M	360 to 460	1½	40	3/7	49	32 to 70	116	99 to 240
C	M	176 to 280	1½	40	0/7	25	17 to 34	48	29 to 88
C	M	260 to 340	½	30	0/7	14	8 to 20	38	22 to 71
C	F	170 to 230	½	30	0/8	45	15 to 72	146	53 to 232
R	M	300 to 420	1½	20	0/7	22	9 to 35	33	23 to 55
R	M	300 to 420	½	10	0/7	+	+	8½	7 to 12
C	F	128 to 152	½	10	0/7	+	+	6	4 to 8

*Strain R came from the Rockland Company; Strain A is that maintained by the Department of Anatomy, Albany Medical College; Strain C came from our own colony in the Department of Physiology and Pharmacology.

†Each fraction represents the incidence of mortality; the numerator indicates the number of animals that died and the denominator the total number of animals used.

These results emphasize that differences in the strains of rats and especially in weight (age) are important factors in the responses to the barbiturates. It should also be pointed out that recovery, especially with the higher doses, is much faster with compound 897 than with pentothal.

An interesting difference appears (Table V) between the reactions to a single intravenous injection of the three barbiturates: pentobarbital, pentothal, and compound 897. In these observations the ages of the male and female members of a test group are the same as are the doses in terms of weight. The exact dosage chosen, however, is arbitrary, since 30 mg. per kilogram of pentothal is approximately 60 per cent of its M.L.D. and is 75 per cent of the M.L.D. of compound 897. Nevertheless, the recovery from anesthesia is faster with the latter compound.

Pentobarbital and compound 897 show a difference in the reactions between the two sexes, for the female rats exhibit longer periods of deep and total anesthesia than do the male animals, while with pentothal such a differentiation fails to appear. Similar preliminary observations made on dogs disclose that these animals do not exhibit the sexual differences to compound 897 which are noted in rats. The causes for these characteristic responses of male and female rats to pentobarbital and to compound 897 have not been elucidated, except to the extent that Moir[2] found that the discrepancy diminishes but does not disappear after castration of the male rats. Similarly, the injection of testosterone propionate into female rats cuts down the period of anesthesia significantly.[9]

Data from this laboratory on thiamine-deficient dogs[10] suggest that they exhibit a reduced resistance to pentobarbital, in comparison with animals on a complete diet. Such an impairment of resistance previously has been reported for rats.[11] Pentobarbital becomes more effective not only in animals with thiamine deficiency but also in those with a deficiency of ascorbic acid.[12] We may conclude that in addition to age, sex, weight, strain, and species, diet is also an important factor in determining the responses to barbiturates.

TABLE V. INTRAVENOUS INJECTION OF THREE BARBITURATES (1 PER CENT SOLUTION)

COMPOUND	SEX	AGE IN MONTHS	WEIGHT RANGE (GRAMS)	INJECTION TIME IN MINUTES	DOSE MG./KG.	NUMBER OF ANIMALS INJECTED	NUMBER OF ANIMALS SURVIVED	EAR REFLEX ABSENT (MINUTES)		SIDE POSITION MAINTAINED (MINUTES)	
								AVERAGE	RANGE	AVERAGE	RANGE
Pentobarbital	M	2	170 to 230	½	40	9	9	62	40 to 86	118	84 to 155
	F	2	130 to 170	½	40	9	9	100	35 to 130	238	180 to 340
Pentothal	M	3	260 to 310	½	30	9	9	16	8 to 28	32	16 to 81
	F	3	160 to 200	½	30	9	9	15	8 to 20	26	15 to 40
Compound 897	M	3½	260 to 340	½	30	7	7	14	8 to 20	38	22 to 71
	F	3½	170 to 230	½	30	8	8	45	15 to 72	146	53 to 232

SUMMARY AND CONCLUSIONS

Age, weight, sex, strain within a given species, difference in species, diet, and kind of barbiturate, all influence the reaction to this group of drugs.

1. The newborn rat, not more than twenty-four hours old, is more susceptible than is the adult, while rats weighing 50 to 200 grams are less susceptible than are those weighing 201 to 500 grams. It is possible therefore to form a spectrum of resistance lowest in the newborn, maximum in rats weighing from 50 to 200 grams, and intermediate with advance in weight and age.

2. As growth proceeds the first increase in the greater susceptibility of the female to pentobarbital is observed in rats weighing 101 to 200 grams, for in that weight range the female remains in light anesthesia longer, though the lethality is the same for both sexes. In rats weighing 201 to 500 grams and receiving pentobarbital, not only is the duration of narcosis longer in the female but the lethality is greater.

In response to an intravenous injection both pentobarbital and compound 897 produce more pronounced effects in the female than in the male, but pentothal exhibits no sexual difference.

3. The intravenous M.L.D. for male rats weighing 360 to 460 grams is 50 mg. per kilogram for pentobarbital and 40 mg. per kilogram for compound 897.

A more rapid recovery from anesthesia follows the administration of compound 897 than follows dosage with pentothal.

4. Differences in the reaction to barbiturates are noted in three strains of rats.

REFERENCES

1. Fitch, R. H., and Tatum, A. L.: Duration of Action of Barbituric Acid Hypnotics as Basis of Classification, J. Pharmacol. & Exper. Therap. **44**: 325-335, 1932.
2. Moir, W. M.: Influence of Age and Sex on Repeated Administration of Sodium Pentobarbital to Albino Rats, J. Pharmacol. & Exper. Therap. **59**: 68-85, 1937.
3. Nicholas, J. S., and Barron, D. H.: Use of Sodium Amytal in Production of Anesthesia in the Rat, J. Pharmacol. & Exper. Therap. **46**: 125-129, 1932.
4. Etsten, B., Alexander, F. A. D., and Himwich, H. E.: Comparative Toxicity of Pentobarbital in Newborn and Adult Rat, J. Lab. & Clin. Med. **28**: 706-710, 1943.
5. Carmichael, E. B.: Nembutal Anesthesia; Median Lethal Dose of Nembutal (Pentobarbital Sodium) for Young and Old Rats, J. Pharmacol. & Exper. Therap. **62**: 284-291, 1938.
6. Kibler, H. H., and Brody, S.: Metabolism and Growth of Rats, J. Nutrition **24**: 461-468, 1942.
7. Chesler, A., and Himwich, H. E.: Comparative Studies of the Rates of Oxidation and Glycolysis in the Cerebral Cortex and Brain Stem of the Rat, Am. J. Physiol. **141**: 513-517, 1944.
8. Cutting, M., and McCance, R. A.: The Metabolism of Kidney Slices From New-born and Full-grown Rats, J. Physiol. **104**: 288-298, 1946.
9. Kinsey, V. E.: Use of Sodium Pentobarbital for Repeated Anesthesia in the White Rat, J. Am. Pharm. A. (Scient. Ed. **29**: 387-390, 1940.
10. Himwich, W. A., and Himwich, H. E.: The Influence of Some Organs on the Pyruvate Level in the Blood, Am. J. Physiol. **148**: 323-326, 1946.
11. Higgins, G. M., and Mann, F. C.: Relation of Vitamin B to Duration of Anesthesia Induced by Pentobarbital Sodium, S. Clin. North American **23**: 1205-1211, 1943.
12. Richards, R. K., Kueter, K., and Klatt, T. J.: Effect of Vitamin C Deficiency on Action of Different Types of Barbiturates, Proc. Soc. Exper. Biol. & Med. **48**: 403-409, 1941.

LABORATORY METHODS

A SIMPLIFICATION OF THE SEROLOGIC DIAGNOSIS OF SALMONELLA CULTURES

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RECENTLY, the attention of many workers has been drawn to the Salmonella group by numerous publications on the genus. This increased interest in a group recognized for many years is due to several factors: A rise in enteric infections caused by large movements in population and the makeshift living conditions of the war years, improved methods of isolation of the bacteria, and, finally, the fact that rapid and reliable methods of identification of cultures by serologic analysis are now available.

Faced by a greater demand for accurate diagnosis of enteric infections and his own ability to isolate a larger number of intestinal pathogens by the use of improved selective media, the worker in the average laboratory has been placed at a disadvantage by inability to identify the cultures which are isolated. The multiplicity of Salmonella types and the many sera necessary for their exact identification have prevented workers in small laboratories from taking advantage of the newer knowledge of Salmonella serology. Thus, many laboratories still depend upon agglutination tests in which the O and H reactions of the cultures are not distinguished and the phases of the H antigens are not considered. Such a system inevitably leads to errors in diagnosis which may seriously handicap the work of the epidemiologist.

It is highly desirable that exact methods of identification of the principal pathogens among the Salmonella and approximate methods of diagnosis of the remaining types be made available in simplified form. The public health laboratory should be able to identify with certainty *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, *S. typhi*, and *S. sendai*, since these are strictly human types which produce prolonged febrile diseases, result in a relatively high carrier rate, and tend to become endemic in the population. *S. choleraesuis* also should be identified exactly because it frequently produces septicemia with localizations in man. Finally, it is desirable to identify *S. typhimurium* since it occurs more frequently in man than any other of the numerous types which produce acute gastroenteritis.

Laboratories engaged in the study of diseases of the domestic animals should be able to identify the so-called primary salmonelloses; *S. abortus equi* in horses, *S. dublin* in cattle, *S. abortus ovis* in sheep, *S. choleraesuis* in hogs, and *S. pullorum* and *S. gallinarum* in fowls. Here again it is desirable that

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S. typhimurium be identified exactly since it occurs so frequently in all animal species. The remainder of the numerous types which occur in domestic animals may be separated into their respective groups but may not be subjected to exact type determination.

Fortunately, the vast majority of Salmonella strains isolated from man and animals are members of groups A, B, C, D, and E of the Kauffmann-White classification. In 1943 Edwards and Bruner[3] found that 98.3 per cent of 3,019 cultures from man and animals were members of these five groups. In 1946 Seligmann, Saphra, and Wassermann[8] obtained an identical figure in 2,916 cultures from man. In 1947 Bruner and Joyce[1] classified 99.5 per cent of 1,007 cultures from man in these groups. Unpublished data of Edwards and Bruner on 12,000 cultures isolated from man and animals indicate that more than 97 per cent of the strains are found in the five previously mentioned groups. Thus, it is evident that the average laboratory need concern itself only with the identification of Salmonella cultures which are members of five somatic groups.* Other cultures occur so rarely that they may be forwarded immediately to a laboratory which specializes in the study of the genus.

Considering these facts, we propose the following diagnostic scheme for the average routine laboratory. A polyvalent serum which contains agglutinins for antigens I, II, III, IV, V, VI, VII, VIII, IX, X, XII, and XV should be available. This is prepared by injecting a rabbit with a mixture of equal amounts of boiled broth cultures of *S. paratyphi A, S. paratyphi B, S. thompson, S. newport, S. gallinarum, S. anatum,* and *S. newington.* Details of the preparation of polyvalent sera were published in 1942 by Kauffmann.[7]

Five O sera for groups A to E, respectively, should be prepared as follows:

A	I,II,XII	*S. paratyphi A*
B	IV,V,XII	*S. paratyphi B*
C	VI,VII,VIII	*S. thompson* and *S. newport*
D	IX,XII	*S. gallinarum*
E	III,X,XV	*S. anatum* and *S. newington*

Methods of preparation of these sera were given in 1941 by Kauffmann[6] and in 1942 by Edwards and Bruner.[2] It will be noted that groups C_1 and C_2 are represented by only one serum as are also groups E_1 and E_2. In this way the number of necessary sera is reduced.

For the public health laboratory a Vi serum is needed to aid in the identification of *S. typhi* and *S. paratyphi C.* This may be made from a living or an alcohol-treated culture of *S. ballerup* by the method of Edwards and Bruner[2] (1942). Such a serum contains agglutinins for O antigen XXIX and H antigen z_{14}, in addition to Vi, but these antigens occur so rarely that the serum may be used without absorption for the determination of Vi antigen.

*As early as 1930 Kauffmann (Die Technik der Typenbestimmung in der Typhus-Paratyphusgruppe, Zentralbl. f Bakt. [Abt. 1] 119: 152, 1930) proposed a simplified set of 12 OH sera by which the common members of groups A to E could be identified. In 1942 a similar proposal was made by Bornstein (Practical Suggestions for Serological Type Determination of Salmonella Organisms, J. Bact. 44: 719, 1942.)

The public health laboratory should have six H sera as follows:

a	*S. paratyphi A*
b	*S. hvittingfoss, phase 1* (or *S. paratyphi B, phase 1*)
c	*S. choleraesuis, phase 1*
d	*S. typhi*
i	*S. aberdeen, phase 1* (or *S. bonariensis, phase 1*)
1,2,3,5	*S. thompson, phase 2,* and *S. newport, phase 2*

Workers in animal pathology should have the c, i, and 1,2,3,5 sera and, in addition, the following:

e,n,x	*S. abortus equi*
g,p	*S. dublin*

The preparation of H sera was described in 1941 by Kauffmann[6] and in 1942 Edwards and Bruner.[3]

With this modest array of sera and by use of the ordinary biochemical tests it is possible to identify the principal Salmonella types and to group practically all Salmonella cultures.

The polyvalent serum is used in slide agglutination tests, usually in a dilution of 1:5 or 1:10, depending upon the titer of the particular serum. It can be used to test colonies directly from plates of selective media or to test growth from slants of differential media to which colonies are transferred. A bit of growth is mixed in saline on a slide and an equal amount of polyvalent sera added or, if desired, the growth may be emulsified directly in the serum dilution. Agglutination becomes apparent after the slide is tilted back and forth for thirty seconds to one minute. In dealing with cultures from man the Vi serum should be used in conjunction with the polyvalent serum so that O-inagglutinable cultures of *S. typhi* will not be missed. If agglutination is not obtained thus with growth from any of the suspicious colonies, those which yield growth resembling Salmonella on differential media should be carried through the usual biochemical tests to eliminate the presence of rare Salmonella types. If the biochemical tests indicate that the organisms are Salmonella, they should be sent to a center for identification.

If agglutination occurs in Vi serum, *S. typhi* should be suspected. In the event that agglutination occurs in the polyvalent serum but in Vi serum, it is probable that a Salmonella other than *S. typhi* is present. Cultures which agglutinate in the polyvalent serum should be tested in a similar manner with the sera for the various O groups. While some cross-agglutination occurs between the different O groups, the reactions are usually quite distinct so that no difficulty is encountered in determining to which O group a culture belongs. Cultures which agglutinate in Vi serum should be heated in saline at 100° C. for a few minutes and tested with the O sera. Thus, the Salmonella strains can be divided into their respective O groups. Cultures which are initially agglutinable by Vi serum after heating should fall into group C if they are *S. paratyphi C* and into group D if they are *S. typhi.* If no agglutination occurs in the O sera after heating, the heated culture should again be tested in Vi serum.

If agglutination still occurs, it may be concluded that the culture contains XXIX antigen and is not a member of any of the common groups. Very rarely rough cultures of *S. typhi* are isolated which will not agglutinate with group D serum after heating. Thus, it is necessary to determine by biochemical methods whether cultures, which in the living state agglutinate in Vi serum but after heating are inagglutinable by group C, group D, and Vi sera, are in reality rough cultures of *S. typhi.*

After the O group of the culture is determined its H antigens should be examined. This may be done by testing growth from an agar slant on a slide with H sera which are diluted 1:50 or 1:100. An alternate method is that recommended in 1942 by Edwards and Bruner,[3] in which an infusion broth culture is diluted with an equal amount of saline containing 0.6 per cent formalin and tested with appropriate sera at 50° Centigrade. Only one dilution (1:1000) is used and results are read after incubation for one hour in a water bath.

In dealing with cultures from man the group A cultures should be tested with serum a to assure that they are *S. paratyphi A,* the only known type in that group. In addition, they should be subjected to the differential tests, nonfermentation of xylose, and inability to utilize tartrates and citrate. The majority of *S. paratyphi A* strains produce little or no H_2S.

Group B cultures should be tested with H sera, b, i, and 1,2,3,5. In the event that agglutination occurs in b or i serum, the culture is almost certainly *S. paratyphi B* or *S. typhimurium,* respectively. Diphasic cultures often occur only in one phase when recently isolated. If phase 2 only is present, it is necessary that phase 1 be obtained to identify the culture. Thus, if the culture is agglutinated only by 1,2,3,5 sera, it should be planted on a Gard plate (Kauffmann,[6] 1941) with 1,2,3,5 serum or placed in a tube containing 2 or 3 c.c. of semisolid medium to which a loopful of that serum has been added. Phase 1 will migrate through the medium and can be isolated from the spreading growth. Useful modifications of the Gard technique have been described by Edwards and Bruner[2] (1942), Hajna[4] (1944), and Juenker[5] (1946). After phase 1 is obtained it can be determined whether the culture is *S. paratyphi B, S. typhimurium,* or some other type within group B.

Group C cultures should be tested with c and 1,2,3,5 sera. If agglutination occurs in c serum, the culture may be identified as *S. paratyphi C* or *S. choleraesuis* depending upon its biochemical reactions. *S. paratyphi C* usually ferments arabinose and always ferments trehalose promptly whereas *S. choleraesuis* attacks neither of these sugars. *S. paratyphi C* ferments dulcitol promptly while *S. choleraesuis* gives delayed or negative reactions in dulcitol media. If phase 2 only is present, phase 1 should be isolated as previously described and tested with c serum. It should be remembered that the majority of cultures of *S. choleraesuis* belong to the Kunzendorf variety in which phase 1 is more or less completely suppressed, so that any group C culture which agglutinates 1,2,3,5 serum, fails to ferment arabinose and trehalose, and gives a negative or delayed result in dulcitol may be reported as *S. choleraesuis.*

Group D cultures should be tested in a, d, and 1,2,3,5 sera. Cultures which agglutinate only in d serum, which are anaerogenic, and which possess the usual biochemical attributes of *S. typhi* may be reported immediately. The organism will have been examined already for the presence of Vi antigen so that its identity is fairly well established. Some recently isolated cultures of *S. typhi* are very poorly motile and may fail to flocculate in d serum. Such cultures can be typed as *S. typhi* provided they possess IX, XII, and Vi antigens and the biochemical properties of *S. typhi*.

Identification of *S. sendai* depends upon the recognition of both phases and biochemical examination. It is necessary that the organism agglutinate both in a and 1,2,3,5 sera. If only one phase is present the second must be isolated as previously described. Further, this type is H_2S and citrate negative and gives a negative result in Stern's glycerol-fuchsin broth. Arabinose is fermented immediately but xylose and sorbitol give delayed reactions. Cultures with similar antigens but different biochemical reactions belong to another type. *S. sendai* occurs frequently only in the Orient.

Cultures which belong to group E are not examined further but are recorded simply as group E Salmonella.

Cultures of animal origin are treated in much the same manner as human cultures. Group A cultures are known to occur only exceptionally in animals and need not be considered. Group B cultures are tested with e; e,n,x; i and 1,2,3,5 sera. *S. abortus equi* should agglutinate only with e,n,x serum and when planted in semisolid medium containing e,n,x serum it should fail to spread since this type is monophasic. Both phases of *S. abortus ovis* should be recognized. This type should agglutinate in e and 1,2,3,5 sera. Further, the organism should fail to ferment trehalose. It is characterized by a more delicate growth on agar media than that exhibited by most Salmonella. *S. typhimurium* is identified as previously described.

Group C cultures are treated as are human strains of group C in order to identify *S. choleraesuis*.

Motile strains of group D are tested with g,p serum to determine whether they are *S. dublin; S. enteritidis* also will agglutinate with this serum, but typical Dublin strains can be differentiated from *S. enteritidis* by the fact that *S. dublin* gives a negative or delayed test with arabinose while *S. enteritidis* ferments the sugar promptly.

Nonmotile strains of group D are probably *S. gallinarum* or *S. pullorum*. Such cultures which are anaerogenic and which promptly ferment dulcitol and maltose may be classified as *S. gallinarum*. *S. pullorum* is usually aerogenic but anaerogenic strains occur. Typical cultures ferment neither maltose nor dulcitol although maltose-fermenting strains occasionally are found. Nonmotile cultures which fail to ferment dulcitol may be classified as *S. pullorum*. This type is also characterized by its sparse growth on artificial media.

In summary, it is hoped that through the adoption of a system similar to that previously described, a more satisfactory diagnosis of Salmonella types may be achieved in the routine laboratory. Such laboratories should not attempt to type every Salmonella culture but should identify exactly only the more

important types. The remaining cultures should be designated "Salmonella, type undetermined" and sent to a center for identification. The adoption of such a system will permit the rapid diagnosis of 98 per cent of Salmonella infections. At present many laboratories still rely on ordinary agglutination tests with sera of doubtful authenticity and specificity and upon biochemical reactions. Reports on Salmonella cultures are therefore often long delayed and inaccurate.

It is realized that this proposal is not strikingly original and that any proposal short of complete typing must be a compromise. However, the system is flexible and can be modified to suit the needs of the individual. The methods proposed here are based upon our experience, with a view toward the employment of a minimal number of sera.

REFERENCES

1. Bruner, D. W., and Joyce, B. J.: Salmonella Types Encountered by the 15th Medical General Laboratory, Am. J. Hyg. **45**: 19-24, 1947.
2. Edwards, P. R., and Bruner, D. W.: The Serological Identification of Salmonella Cultures, Ky. Agr. Exp. Sta. Circular 54, 1942.
3. Edwards, P. R., and Bruner, D. W.: The Occurrence and Distribution of Salmonella Types in the United States, J. Infect. Dis. **72**: 58-67, 1943.
4. Hajna, A. A.: Use of a "U" Tube for the Isolation of Monophasic Varieties From Diphasic Salmonella Cultures, J. Bact. **48**: 609-610, 1944.
5. Juenker, A. P.: A Rapid Method of Phase Isolation in Salmonella Cultures, J. Bact. **52**: 609, 1946.
6. Kauffmann, F.: Die Bakteriologie der Salmonella-Gruppe (Typhus-Paratyphus-Enteritis-Gruppe), Copenhagen, 1941, Ejnar Munksgaards Forlag.
7. Kauffmann, F.: Ueber polyvalente Immunseren zur Salmonella-Diagnose, Acta path. et microbiol. Scandinav. **19**: 248-261, 1942.
8. Seligmann, E., Saphra, I., and Wassermann, M.: Salmonella Infections in the U.S.A., J. Immunol. **54**: 69-87, 1946.

THE PROTHROMBIN TIME AND VARIABLES IN THE METHOD

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THE prothrombin test as originally devised by Quick[1] and subsequently modified by Link,[2] Shapiro,[3] Brambel,[4] and others is steadily becoming more important in clinical medicine, particularly as a guide to dicumarol therapy. Accurate, consistent results are imperative if patients are to be adequately treated yet not unnecessarily subjected to the danger of serious hemorrhage. Indeed, if dependable prothrombin values are not obtainable, dicumarol therapy must be discarded.

Our own early experience and observations of some other laboratories lead us to believe that prothrombin results are very often dangerously inaccurate. In the course of our work some factors causing variations have been found. Some have not been mentioned previously and others have received too little emphasis. It is hoped that the recognition of some of the pitfalls may be of value to others and of help in the development of a more reliable technique.

THE PROTHROMBIN TEST—DESCRIPTION OF TECHNIQUE

All one-stage prothrombin tests used today are based on the original method of Quick. We have followed the Brambel modification of the Quick test because it is more accurate in determining early changes in prothrombin content. This is particularly true between 35 and 100 per cent, where the Brambel dilution curve rises more sharply than do either the dilution curves of Quick or Link and Shapiro (Fig. 1).

Regardless of the method used, each laboratory should determine its own dilution curve in order to report accurately in per cent of prothrombin. Variations in the strength of the thromboplastin and other factors that will be considered later make it impossible to read results from dilution curves that have been made in other laboratories. In the following outline of the method, we follow closely the Brambel technique[4] and note its variations from Quick.

Thromboplastin is prepared from rabbit brain according to the method described by Brambel.[4] It is then kept in a desiccator at 4° C. or sealed in vacuum bottles and kept in the refrigerator. When ready for use, the dried brain containing the thromboplastic substance is removed from the vacuum tubes or the desiccator and ground to a fine powder with a pestle and mortar. (With Quick's method the thromboplastic substance is used without any further grinding.) Then 250 mg. of this powder are suspended in 5.0 c.c. of normal saline solution (0.85 per cent) containing 0.1 c.c. of a 0.1 molar solution of sodium oxalate. After the test tube is thoroughly shaken to insure uniform

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distribution of the particles, it is placed in a water bath maintained at 50° C. for a period of just fifteen minutes. While in the water bath, the test tube is vigorously shaken for ten seconds every two minutes. It is important that the material be shaken regularly and consistently at the two-minute period, as the amount of agitation will change the prothrombin times obtained. Additional shaking will cause times to increase.

At the end of the incubation period the material is put in a centrifuge, and revolved at 500 r.p.m. for just twenty seconds. This will give a milky supernatant fluid which may then be pipetted off and used for testing.

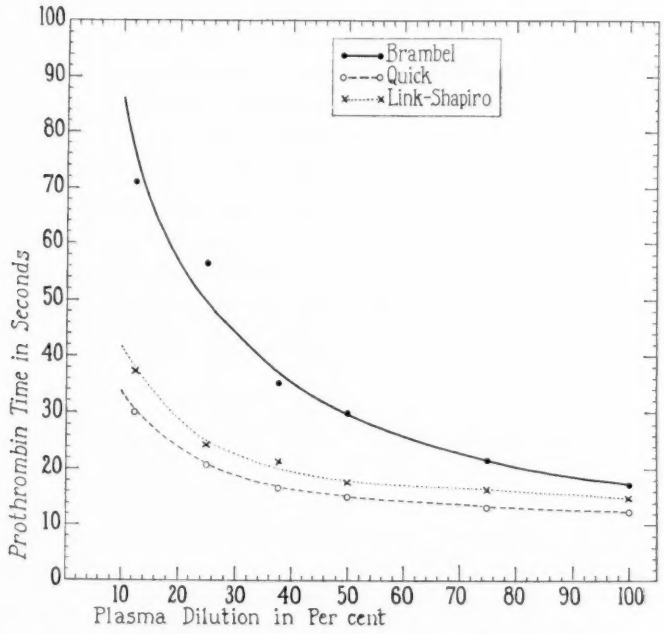

Fig. 1.—Dilution curves obtained by three different methods.

Quick's method of preparation of thromboplastin differs from the previously described method of Brambel only in the following ways: (1) There is no grinding of the acetone-washed, dried brain before adding the normal saline-sodium oxalate mixture; (2) during the period of incubation the only agitation is that produced by the occasional blowing through the mixture with a pipette; and (3) 300 instead of 250 mg. of thromboplastic material are incubated in 50 c.c. of saline-oxalate mixture. This change of amount does not appear to be important, as an excess of thromboplastin is used in both methods.

Plasma for prothrombin determination is obtained by mixing 4.5 c.c. of venous blood with 0.5 c.c. of 0.1 molar sodium oxalate. This is centrifuged at 1,500 r.p.m. for twenty minutes.

One-tenth of a cubic centimeter of plasma is then placed in an 8 by 100 mm. test tube and to this is added 0.10 c.c. of thromboplastin solution. The two are thoroughly mixed and placed in a constant temperature water bath

of 37° C. for two minutes. One-tenth of a cubic centimeter of 0.025 molar calcium chloride, which has previously been allowed to stand at 37° C., is then drawn into a 1.0 c.c. pipette calibrated in 0.10 c.c. graduations to the tip. The calcium chloride is quickly blown into the plasma-prothrombin mixture and at the same moment a stop watch is started. A foot switch for the operation of the watch as described by Shapiro is helpful.[3]

In introducing the calcium chloride into the plasma-prothrombin mixture, it is important to keep several points in mind. The transfer must be made quickly, but care must be taken so that the solutions will not remain on the side of the tube and prevent complete mixing. The pipette used for calcium chloride must be very carefully wiped before it is used again. If this is not done, small drops of the prothrombin-plasma mixture may adhere to the sides of the pipette and contamination of the calcium chloride solution will result, invalidating further tests. A fresh pipette may be used for each transfer of the calcium chloride, but this does not seem necessary if due care is taken.

Once the solutions are mixed by shaking, the mixture is held in the water bath for ten seconds. The tube is then lifted out and gently tilted back and forth until the solution abruptly gels. When coagulation first begins, the stop watch is stopped and the time recorded to the nearest one-tenth of a second. All runs should be done in duplicate and should check within a second for undiluted plasmas.

EXAMINATION OF POSSIBLE VARIABLES IN THE METHOD

Stability of Thromboplastin in Dry State.—As others have reported, we also have found that the stored thromboplastin loses its potency when exposed to air but that the temperature factor is not so important. According to Quick,[5] when thromboplastin is stored at 4° C. in vacuum containers, potency is maintained over a period of years.

We have tested the same dried thromboplastic substance, prepared according to the Brambel method, over a period of five and one-half months and have found no decrease in the strength. The figures in Table I give the prothrombin times obtained on the same normal individuals over a period of months. In this experiment three separate batches of thromboplastin, kept continuously in a desiccator at 4° C., were used for testing.

TABLE I. STABILITY OF DRIED THROMBOPLASTIN STORED IN DESICCATOR AT 4° C. (FIGURES GIVE PROTHROMBIN TIME IN SECONDS)

	DATE TESTED	THROMBOPLASTIN		
		A	B	C
Normal plasma (G. C.)	June 24	17.4		
	July 24		17.2	
	Aug. 19	17.6	17.3	17.4
	Dec. 11	17.2	16.8	17.2
Normal plasma (J. M.)	June 29	17.0		
	July 13		17.1	
	Aug. 16			17.1
	Dec. 11	16.9	16.5	16.8

Stability of Thromboplastin in Solution.—It is of some importance to know how long this thromboplastic solution maintains its strength, for time and material may be saved if its stability is such that it may be used for more than one series of tests. In Tables II and III the results of daily tests on the same individuals are shown. The thromboplastic solutions were made up fresh on the first day and kept in the refrigerator at 2° C. for the duration of the experiment. Bloods were drawn at the same time each day on the normal individuals tested, and in all respects the tests were done in the same manner each day.

In Table II are shown the daily times obtained using thromboplastin prepared in our laboratory from the acetone extraction of rabbit brain. When not used for testing, it was kept in the refrigerator. It will be noted that there is a change in potency on the sixth day. This is more noticeable in the first three cases which were done some months before the last four by a different technician and with a different lot of thromboplastin. It would appear justifiable on the basis of these data to use such thromboplastic solutions for at least four days before discarding them.

TABLE II. THROMBOPLASTIN (BRAMBEL), +2° C.
(PROTHROMBIN TIME IN SECONDS)

CASE	FIRST DAY	SECOND DAY	THIRD DAY	FOURTH DAY	FIFTH DAY	SIXTH DAY	SEVENTH DAY	EIGHTH DAY	NINTH DAY
1	17.2	17.3*	17.4	17.4*	17.4	23.8	24.8		
2	17.1	17.4	17.2	17.5	17.5*	24.8	24.8†		
3	17.2	17.2	17.3	16.0	17.4*	23.9	24.8†		
4	17.7	17.5	17.8	18.4	18.1	21.0	22.0		
5	17.9	18.1	18.2	17.7	19.0	19.1	20.4†		
6	19.0	18.1	18.4	18.0	18.1	18.7	19.1	19.1	19.0
7	20.0	18.9	17.4	17.6	17.7	19.0	21.2	21.2	21.0
Average	18.0	17.8	17.7	17.5	17.9	21.5	22.4		

*Interpolated.
†Extrapolated.

TABLE III. POTENCY THROMBOPLASTIN (MALTINE) IN SOLUTION, +2° C.
(PROTHROMBIN TIME IN SECONDS)

NORMAL	FIRST DAY	SECOND DAY	THIRD DAY	FOURTH DAY	FIFTH DAY	SIXTH DAY	SEVENTH DAY	EIGHTH DAY	NINTH DAY
1	16.6	16.0	17.4	18.7	17.6	17.6	19.2	19.1	19.6
2	16.3	16.1	15.9	18.4	18.0	17.4	21.1	18.0	18.0
3	16.7	17.0	16.7	18.3	17.7	17.6	18.9	18.5	21.0
4	16.1	16.6	16.0	18.4	18.0	17.5	19.8	18.8	19.0
5	13.7	16.3	16.5	18.0	17.6	17.5	18.5	18.3	18.1
Average	15.9	16.4	16.5	18.4	17.8	17.5	19.5	18.5	19.1

In many laboratories it is not feasible to prepare thromboplastin from rabbit brain, and the use of a commercial product is necessary. We have therefore extended our observations to one, frequently used, ready-made thromboplastin (Maltine). The experiment was carried out in the same manner as previously described. In this instance there is a significant loss of strength on the fourth day, but values are consistent for the first three days (Table III).

Calcium Chloride Solution.—We have used a 0.025 molar concentration of calcium chloride. This is the same strength solution as is used by Shapiro and Brambel. Quick also previously used this solution, but has more recently

changed to a 0.020 molar strength. This may account in part for shorter times observed when using the Quick technique, as the calcium chloride concentration is a critical factor in this test.

Pohle and Stewart[6] showed that even small variations of the calcium chloride had a distinct effect on the prothrombin time. As the calcium chloride strength was reduced to about 0.01 molar the prothrombin times became shorter. Below this level there was a rapid lengthening. We have confirmed these observations, as is shown in Fig. 2. Actually, other molarities than the usual 0.025 molar might be adopted if a dilution curve for that solution were set up and percentage values then derived from that curve. The important consideration is that the calcium chloride solution be consistently of the same strength.

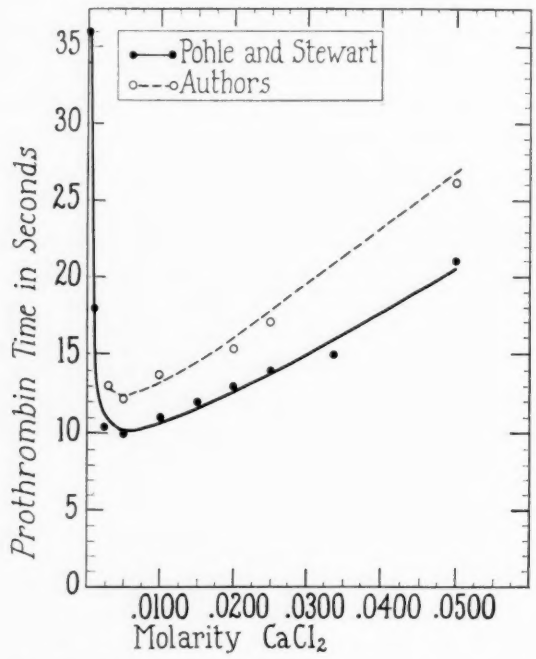

Fig. 2.—Effect of calcium concentration on prothrombin time.

Solutions should be made up accurately and in large enough volume to obviate the necessity of frequent preparation of new stock solutions, which might lead to error. As anhydrous calcium chloride is most hygroscopic, its weight will change even as it is being removed from the container for weighing. For critical work a final chemical analysis of the solution is desirable.

Plasma—Collection of the Blood.—Plasma is obtained by mixing 4.5 c.c. of blood with 0.5 c.c. of 0.1 molar sodium oxalate. The mixture is then centrifuged for twenty minutes at 1,500 revolutions per minute. It is important that the vein be entered cleanly and a perfectly dry syringe be used, as slight clotting or hemolysis of the blood will cause shortening of prothrombin times (Figs. 3 and 4).

Plasma Stability.—It is usually assumed that the prothrombin test will be run soon after the blood is drawn, but there is little specific information on the stability of the plasma at various temperatures. Link[2] reported that normal rabbit plasma was constant in prothrombin content for several hours at room temperature but that bloods of animals eating spoiled sweet clover (dicumarol equivalent) were likely to change in value after standing three or four hours. Campbell and associates[7] have found that no appreciable error was introduced in keeping plasma obtained from normal rabbits for a week at 0° Centigrade.

In Tables IV to VII tests have been run on both abnormal and normal plasmas, kept for varying times at different temperatures. It will be seen that although there is a more rapid deterioration of prothrombin at high temperatures than at lower ones, prothrombin stability does not vary directly nor consistently with temperature change.

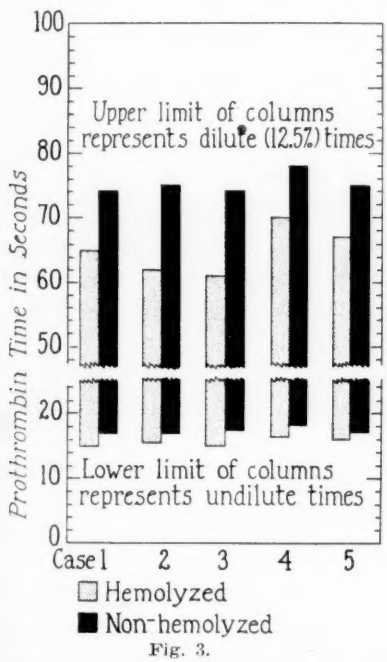

Fig. 3.

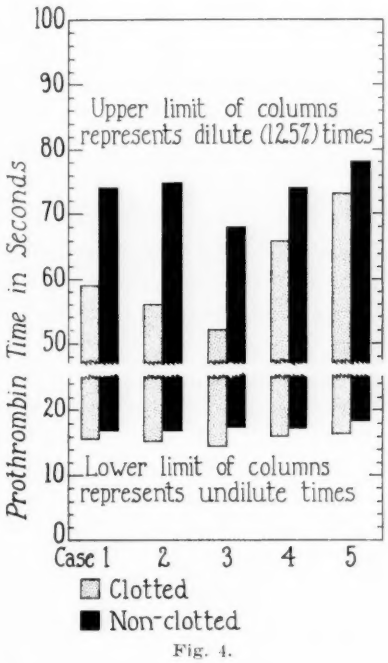

Fig. 4.

Fig. 3.—Effect of hemolysis on prothrombin time.
Fig. 4.—Effect of clotting on prothrombin time.

In Table IV is shown the loss of prothrombin potency when plasmas are kept refrigerated at 1 to 2° Centigrade. Bloods were drawn at 9:30 A.M., and the plasma was immediately separated by centrifuging and placed in the icebox. The first determinations were made one hour after bloods were drawn and the second test four hours after obtaining the specimens. There is no significant change up to four hours, but after seven hours the three normals do show definite lengthening of times. At ten hours all show evidence of weakening of the prothrombin strength. It is obviously impossible to keep plasmas overnight in a refrigerator and still obtain accurate results.

TABLE IV. PLASMA STABILITY, 1 TO 2° C.
(PROTHROMBIN TIME IN SECONDS)

CASE	TIME AFTER DRAWING OF BLOOD			
	1 HOUR	4 HOURS	7 HOURS	10 HOURS
1	18.5	17.8	17.8	20.5
2	29.8	27.0	29.0	33.0
3	33.0	33.1	32.4	35.3
4	29.9	31.8	32.3	37.2
5	39.7	37.0	40.0	44.1
6*	15.5	15.5	17.8	19.5
7*	14.8	15.6	17.1	17.8
8*	15.1	15.2	17.7	18.2

*Normals.

TABLE V. PLASMA STABILITY, 21° C. (70° F.)
(PROTHROMBIN TIME IN SECONDS)

CASE	TIME AFTER DRAWING OF BLOOD				
	1 HOUR	2.5 HOURS	4 HOURS	5 HOURS	6 HOURS
1*	15.8	15.7	15.5	16.1	16.1
2*	15.8	15.9	15.7	15.9	16.2
3	24.3	23.9	23.2	24.8	26.3
4	18.7	18.8	19.6	20.9	22.0
5	29.0	28.7	29.8	31.6	32.6
6	28.0	27.4	27.1	27.7	27.4
7	27.3	27.4	26.8	28.6	31.1
8	30.2	30.1	31.9	32.2	34.9

*Normals.

In a similar manner blood samples were obtained and the plasmas allowed to remain at a room temperature of 21° C. (70° F.). Loss of potency was not greatly accelerated as compared to plasmas kept in the icebox, but in five hours' time some samples gave evidence of slight loss of prothrombin activity. In six hours five of the eight gave longer values (Table V).

A series run during the summer months at a room temperature of 29° C. (89° F.) showed no significant variation at five and one-half hours but considerable deterioration of prothrombin activity after six and one-half hours (Table VI). It appears, therefore, that with the room temperatures usually

TABLE VI. PLASMA STABILITY, 29 TO 31° C. (84 TO 88° F.)
(PROTHROMBIN TIME IN SECONDS)

CASE	TIME AFTER DRAWING OF BLOOD							
	1.5 HOURS	3.5 HOURS	4.5 HOURS	5.5 HOURS	6.5 HOURS	7.75 HOURS	9.25 HOURS	10.25 HOURS
1*	17.0	16.9	17.7	16.5	21.6	26.8	21.9	26.1
2*	45.4	45.7	46.8	43.8	58.0	63.0	53.0	61.0
3	26.6	25.0	27.9	24.0	32.6	33.7	32.5	39.1
4	17.7	17.6	18.0	17.6	22.7	26.4	28.0	24.7

*Normals.

TABLE VII
(PROTHROMBIN TIME IN SECONDS)

CASE	45 MINUTES AFTER DRAWING BLOOD (TEMPERATURE 21° C.)	1 HOUR LATER (PLASMA KEPT AT 37° C.)
1*	14.8	18.9
2*	15.1	18.3
3	18.5	22.8
4	29.8	36.1
5	37.9	43.7

*Normals.

encountered (70 to 84° F.) there is little variation in rate at which prothrombin activity is lost.

All specimens should be run as soon as possible after the bloods are drawn, and any plasma kept longer than five hours either at room or icebox temperatures cannot be interpreted with accuracy.

When the temperature is raised to 37° C., loss of potency is much more rapid, as is shown in Table VII. This sudden rise in rate of prothrombin destruction is somewhat surprising in view of results noted at lower temperatures. We are unable to explain this variation in action. Perhaps prothrombin loss is due to the action of enzymes present in the plasma or it may be a result of bacterial activity. No attempt has been made to run these tests under strict aseptic conditions.

Plasma—Time of Taking Blood Samples.—Meyers and Poindexter,[8] using the dilute prothrombin method of Shapiro, showed that patients with coronary arteriosclerosis had a slightly shorter prothrombin time in the evening and early morning hours. Levan[9] states that "determinations after breakfast were considerably higher in per cent prothrombin than fasting specimens." That is, the prothrombin times after breakfast were shorter.

We have run serial determinations throughout the day on ambulatory patients. Since other work being carried on in the laboratory at this time necessitated the use of the Link-Shapiro method, it was employed in these particular determinations.

The first specimens were taken at 8:30 A.M. and breakfast was eaten immediately. A second blood was drawn at 10:30 A.M., approximately two hours after breakfast. Lunch was eaten at 11:45 A.M., and a midafternoon sample was taken at 3:00 P.M. Supper was served at 5:00 P.M., and the fourth and last blood was drawn at 11:00 P.M. We did not find any significant difference between bloods drawn before and after breakfast, and it would not appear necessary to insist on fasting blood samples. There is, however, consistent lengthening of the prothrombin times of the 3:00 P.M. samples when these are compared to morning samples. The specimens drawn at 11:00 P.M. have somewhat shorter times than those in midafternoon but are longer than morning tests (Table VIII).

TABLE VIII. DIURNAL VARIATION (MALTINE THROMBOPLASTIN) (TIME IN SECONDS)

CASE	8:30 A.M.	10:30 A.M.	3:00 P.M.	11:00 P.M.
1	14.5	15.0	15.5	15.1
2	15.5	16.0	16.5	16.2
3	15.3	15.2	16.2	15.3
4	15.2	15.2	16.8	15.9
5	15.6	15.6	18.0	17.1
6	15.2	16.2	17.2	16.2
Average	15.2	15.5	16.7	16.0

Meals at: 8:40 A.M., 11:45 A.M., and 5:00 P.M.

For maximum accuracy it is advisable to take blood samples at the same time each day. It may well be that patients on dicumarol show even wider diurnal fluctuations, but this is impossible to demonstrate, as any such change would be obscured by fluctuations in levels due to the action of the drug itself.

TABLE IX

TYPE OF MEAL	TIME OF SPECIMEN	PROTHROMBIN TIME (SECONDS) PATIENT 1	PATIENT 2	PATIENT 3
Breakfast with 20 Gm. fat	Fasting	17.5	17.0	17.4
	Two hours after meal	14.7*	16.9	16.9
Breakfast with 50 Gm. fat	Fasting	17.6	17.8	17.5
	Two hours after meal	17.1	17.4	16.9

*Hemolyzed blood.

It may be that the degree of lipemia of the blood following meals may account for a slight diurnal variation. Pohle and Stewart,[6] using the Quick method, found a shortening of times after a meal heavy with fat. We have not been able to show any difference in the undiluted times on bloods taken (1) two hours after an average breakfast containing 20 to 30 Gm. of fat and (2) those taken before and two hours after a breakfast containing 50 Gm. of fat (Table IX).

Variations in the Running of the Prothrombin Test (Quick Method or Brambel Modification).—

Water Bath Temperature: As shown in Tables X and XI, no great error is introduced if the temperature of the water bath is changed from 35 to 40° Centigrade.

TABLE X. EFFECT OF TEMPERATURE CHANGE OF WATER BATH ON PROTHROMBIN TIMES (BRAMBEL TECHNIQUE AND THROMBOPLASTIN PREPARED IN OUR LABORATORY) (TIME IN SECONDS)

TEMPERATURE	UNDILUTED PLASMA CASE 1	2	3	4	12.5% SALINE DILUTION CASE 1	2	3	4
35° C.	16.5	17.0	26.8	31.4	67.0	78.0	177	307
37.5° C.	16.6	17.0	27.0	31.2	66.0	79.0	181.5	310
40° C.	15.5	16.9	25.7	30.5	66.5	71.3	181.5	306

TABLE XI. EFFECT OF TEMPERATURE CHANGE IN WATER BATH (LINK-SHAPIRO TECHNIQUE WITH MALTINE THROMBOPLASTIN) (TIME IN SECONDS)

TEMPERATURE	UNDILUTED PLASMA CASE 1	2	3	4	12.5% SALINE DILUTION CASE 1	2	3	4
35° C.	14.3	14.9	23.7	26.2	32.8	34.8	55.0	64.5
37.5° C.	14.4	15.0	23.3	25.5	31.9	35.3	58.5	62.8
40° C.	14.0	14.5	21.8	25.0	31.6	34.1	54.5	65.0

TABLE XII
(PROTHROMBIN TIME IN SECONDS)

CASE	TILT TUBE METHOD	WIRE LOOP METHOD
1	21.9	21.6
2	15.7	16.1
3	24.9	24.6
4	15.9	16.7
5	24.5	24.9
6	27.2	26.8
7	15.3	15.2
8	15.0	14.5
9	16.1	15.8
10	25.2	24.4

The same lack of variation in times with changes in temperature of the water bath is also found when the Link-Shapiro method is used with maltine thromboplastin.

Use of Tilt Tube or Wire Loop Method: Some workers determine the time of clotting by passing a thin wire loop through the solution and noting when the fibrin first adheres to the wire. We have run parallel determinations on the same plasmas using this wire method and the tilt-tube technique. No consistent difference is apparent in the results shown in Table XII.

Order of Mixing Reagents: There is another variation in the running of the test that does alter the results. In the Link-Shapiro method the calcium chloride and thromboplastin are first mixed together and finally 0.10 c.c. of plasma is added just as the stop watch is started. In the Quick and Brambel methods the thromboplastin and plasma are first mixed and the calcium chloride is added last. We have found that this minor change in technique will give a consistent difference in results. When the calcium chloride is first mixed with thromboplastin and the plasma is added last, the times are shorter than when there is premixing of plasma and thromboplastin with a final addition of calcium chloride. The results of the two methods run on twelve consecutive plasmas are shown in Fig. 5. Both methods give reproducible times, but it is apparent that the same technique must be consistently followed.

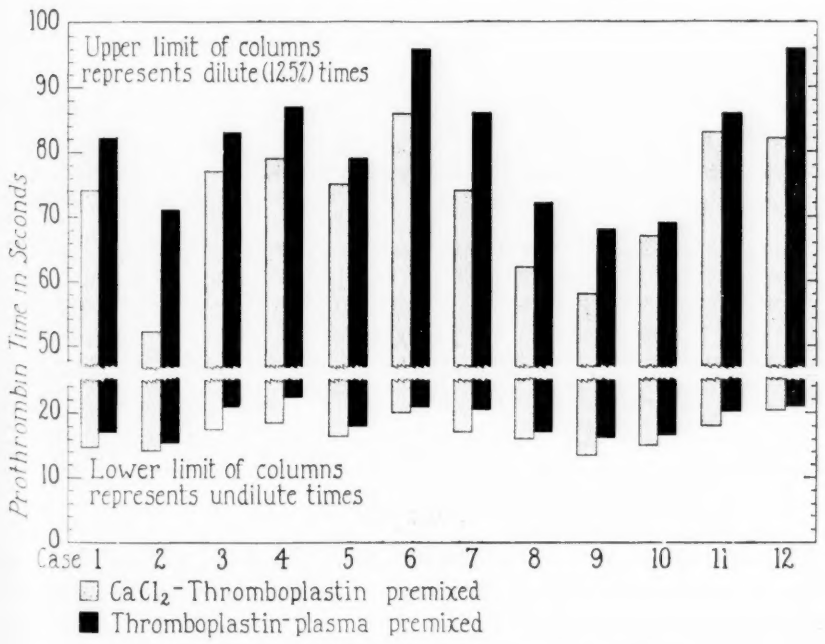

Fig. 5.—Effect of changing the order of mixing the reagents.

RANGE OF NORMAL

Abnormal findings cannot be properly evaluated until the range of normal is known. Too few data have been published on normal prothrombin times as

TABLE XIII. REPEATED DETERMINATIONS ON FOUR NORMAL MEN AND WOMEN

CASE	SEX	NUMBER OF DETERMINATIONS	UNDILUTE			DILUTE		
			MEAN	S.D.	(RANGE)	MEAN	(S.D.)	RANGE
1	M	9	17.1	± .3	16.6 to 17.6	77	±7	69 to 101
2	M	11	17.3	± .7	16.1 to 18.6	91	±8	74 to 120
3	F	10	17.0	± .5	15.8 to 17.5	71	±2	67 to 74
4	F	10	17.1	±0.5	16.0 to 17.5	75	±8	66 to 96

measured by the various methods, and care must be taken lest times be reported as "abnormal" when they really come within normal range.

The figures in Table XIII will serve to show the variations possible in this method in a group of healthy individuals. Tests were done on two normal men and two normal women at various intervals over a period of several weeks. The maximum range in the undiluted test was 2.5 seconds and in the 12.5 per cent saline dilution 46 seconds.

In Table XIV are shown the variations in times obtained in a group of ninety-three normal people, fifty-eight women and thirty-five men. Most of these were nurses and medical students between the ages of 20 and 30 years of age. Three different batches of thromboplastin were used. It will be noted that while the standard deviation for the undilute plasma is only 0.6 seconds, the range of normal is three seconds. Dilute times are much less consistent, with a standard deviation of ten seconds and a range of normal from 56 to 115 seconds. Therefore, moderate variations in the dilute times, as obtained by this method, are of doubtful significance. Work now in progress leads us to believe that the practice of diluting the plasma to 12.5 per cent with saline, as is advocated by those using the "dilute method," leads to the introduction of uncontrolled variables and gives inaccurate results. As a matter of fact, the use of saline in making a dilution curve is probably not entirely sound.

TABLE XIV. RANGE OF NORMAL IN HEALTHY INDIVIDUALS
(NINETY-THREE NORMAL INDIVIDUALS)

BATCH OF THROMBOPLASTIN	MEN	WOMEN	TOTAL	UNDILUTE MEAN	UNDILUTE (S.D.)	UNDILUTE RANGE	DILUTE MEAN	DILUTE (S.D.)	DILUTE RANGE
A	5	12	17	17.2	±.7	15.4 to 18.0	83	±17	61 to 115
B	2	9	11	17.4	±.6	16.9 to 18.3	74	±12	63 to 96
C	51	14	65	17.2	±.6	16.3 to 18.4	74	± 8	56 to 102
Over-all total	58	35	93	17.2	±.6	15.4 to 18.4	76	±10	56 to 115

SUMMARY

1. The prothrombin test as devised by Quick and modified by Brambel has been reviewed in detail and the effect of certain variables examined. These include observations on:

(a) The stability of thromboplastin in solution and the dry state
(b) The effect of changing the molarity of the $CaCl_2$ solution
(c) The effect of hemolysis and clotting of the blood samples
(d) The effect of various temperatures on the plasma prothrombin stability
(e) The effect of changing the order of mixing the reagents

(f) The effect of meals and time of day on resulting times

(g) The effect of water bath temperature in running the test

2. The bloods of ninety-three normal persons were tested for prothrombin content. According to the method described, the following values were obtained:

(a) Mean time for undilute plasma was 17.2 seconds with a standard deviation of 0.6 seconds and a range of from 15.4 to 18.4 seconds.

(b) Mean time for 12.5 per cent saline dilution of plasma was 76 seconds with a standard deviation of 10 and a range of 56 to 115 seconds.

3. Repeated tests were made on the same normals and mean standard deviation and range were computed for dilute and undilute specimens.

The authors wish to express their appreciation to Dr. Joseph M. Hayman, Jr., for his many helpful suggestions in the preparation of the manuscript.

REFERENCES

1. Quick, A. J.: The Hemorrhagic Diseases and the Physiology of Hemostasis, Springfield, Ill., 1942, Charles C Thomas, Publisher.
2. Link, K. P.: The Anticoagulant From Spoiled Sweet Clover Hay, Harvey Lect. **39:** 162-216, 1944.
3. Shapiro, S.: Hyperprothrombinemia, a Premonitory Sign of Thromboembolization (Description of a Method), Exper. Med. & Surg. **2:** 103-109, 1944.
4. Brambel, C. E.: Thromboplastic Reagent, Arch. Surg. **50:** 137-147, 1945.
5. Quick, A. J.: On the Quantitative Estimation of Prothrombin, Am. J. Clin. Path. **15:** 560-566, 1945.
6. Pohle, F. J., and Stewart, J. K.: A Study of the Quick Method for the Quantitative Determination of Prothrombin With Suggested Modifications, Am. J. M. Sc. **198:** 622-630, 1939.
7. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P.: Studies on the Hemorrhagic Sweet Clover Disease, J. Biol. Chem. **138:** 1-33, 1941.
8. Meyers, L., and Poindexter, C. A.: A Study of the Prothrombin Time in Normal Subjects and in Patients With Arteriosclerosis, Am. Heart J. **31:** 27-32, 1946.
9. Levan, J. B.: Dicumarol Therapy, Ann. Int. Med. **25:** 941-949, 1946.

A METHOD FOR COUNTING BACTERIA IN THE NASAL CAVITY

Application of the Method in Demonstrating the Action of Intranasal Penicillin Administration

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INTRODUCTION

THE usual method of studying the types of bacteria in the nasal cavity is to insert a sterile cotton swab and culture it in liquid or solid media. Such a procedure is satisfactory for a qualitative examination of the nasal flora, as in the diagnosis of diphtheria or other nasal infections, but is totally inadequate for an estimation of the number of bacteria present.

The reasons for the inadequacy of this method for quantitative purposes become readily apparent from the following points of discussion. It is known (Thomson and Thomson[1]), and we have confirmed the fact, that the vestibule of the nostril is colonized with a great number of bacteria, usually air-borne saprophytes, which may contaminate the swab as it is inserted or withdrawn and thus distort the true picture of the types and number of bacteria found within the nasal cavity. The sine qua non of making a quantitative estimate of the number of bacteria from a given liquid or surface at repeated intervals for comparative purposes is the use of a precisely uniform sampling method; this appears to be virtually impossible when inserting a swab into the nasal cavity at repeated intervals. Abnormalities in the topography of the nasal cavity often render many areas inaccessible to the swab. It is entirely possible that nasal bacteria are not uniformly distributed over portions of the cavity which are accessible to the swab. Differences in pressure when inserting the swab may also account for variations in the number of bacteria that are recovered on the swab. All of the bacteria removed by the swab may not be dislodged when it is cultured, especially on a solid medium. These few considerations indicate some of the probable causes why the swab method, which we have tested extensively, is unsatisfactory for obtaining a reliable count of the number of bacteria in the nasal cavity.

Recently, Hamburger and co-workers[2, 3] have devised novel methods for obtaining an estimate of the number of streptococci in the nasal cavity. These consist either of having the subject blow his nose on a sterile cloth which is subsequently cultured in toto and the number of streptococci determined by plate counts of the sample or of pinning a cloth patch to the bed in which the subject sleeps and determining the number of streptococci adhering to the cloth. While both of these methods gave useful information in these workers' experiments, they were admittedly crude and the results were sub-

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ject to considerable variation. So far as we are aware, no other method has been published which gives more reliable knowledge of the number of bacteria in the nasal cavity than those previously mentioned.

In determining the efficacy of intranasally instilled antibacterial agents, it is obviously necessary to employ a method of sampling the nasal flora which will give reasonably reliable and consistent results. For this purpose a simple sampling device and procedure have been developed which are believed to give more reliable quantitative results in obtaining a count of the nasal bacteria than any other procedure thus far recorded in the literature. A description of this sampling device, recommended procedure in sampling, and results obtained are reported in this paper.

SAMPLING DEVICE AND PROCEDURE

The construction of the sampling device which can be readily prepared in most laboratories is shown in Fig. 1. It consists of a test tube 13 by 2.5 cm. with the closed end drawn out to a diameter of 0.5 cm. and cut off. This end of the tube is connected to a glass nasal "olive" by a piece of rubber tubing 20 cm. in length. The opening in the nasal olive measures 1 cm. in diameter at the end inserted into the nostril, 2 cm. at the bulge, and 0.5 cm. at the end connected to the rubber tubing. Variations in these dimensions can be made, of course, but we have found this size satisfactory. The open end of the test tube is plugged with cotton, the nasal olive wrapped in paper, and the entire device sterilized in the autoclave.

In using the sampling device, 20 c.c. of sterile broth or Ringer's solution (the latter being preferred since it is less objectionable to most subjects) are pipetted aseptically into the test tube and the cotton plug replaced. The paper is removed from the olive which is inserted tightly into one nostril with one hand while the test tube is held with the other hand. The head is bent slightly forward and the subject slowly raises the tube, as shown in Fig. 2, until he feels the solution just beginning to trickle down into the throat. At this point he lowers the tube which siphons the solution back into the test tube. This process is repeated two times to insure a thorough washing of the nasal cavity.

The nasal washings thus obtained are run through the nasal olive into a sterile, empty, rubber-stoppered 100 c.c. dilution bottle (French square type) which is shaken twenty-five times to disintegrate clumps of organisms. One cubic centimeter of this suspension as well as 1 c.c. of 1:10 and 1:100 dilutions are plated in blood agar to determine the total number of bacteria per cubic centimeter of the nasal washings. Many variations of this sampling procedure suggest themselves, and we have tested the following: (1) Making five successive washings at five-minute intervals with fresh solutions each time and plating each washing individually; (2) making three successive washings and combining the washings before plating; (3) washing both nostrils and combining the washings; and (4) making washings in the morning and afternoon or only once in the same day.

As a result of these preliminary trials, the following procedure was chosen finally as giving the most reliable results for testing the antibacterial action of intranasally instilled agents. In order to determine the average daily normal count for control purposes, at the same time each day for a week or more preceding the use of the agent, a sample is obtained from each subject as follows: The nasal olive is inserted into one nostril and the irrigating solution allowed to flow in and out of the nasal cavity twice; then the olive is inserted into the other nostril and the process repeated. Two additional sampling devices, each filled with 20 c.c. of solution, are used successively in the same way. All three washings (60 c.c.) are then pooled, shaken, and cultured as described previously.

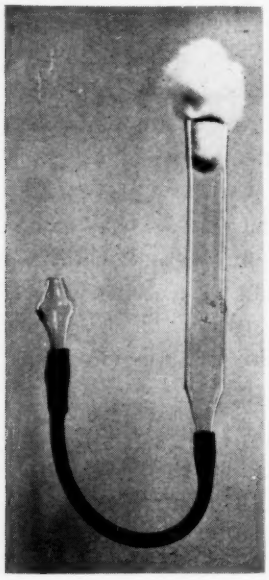

Fig. 1.

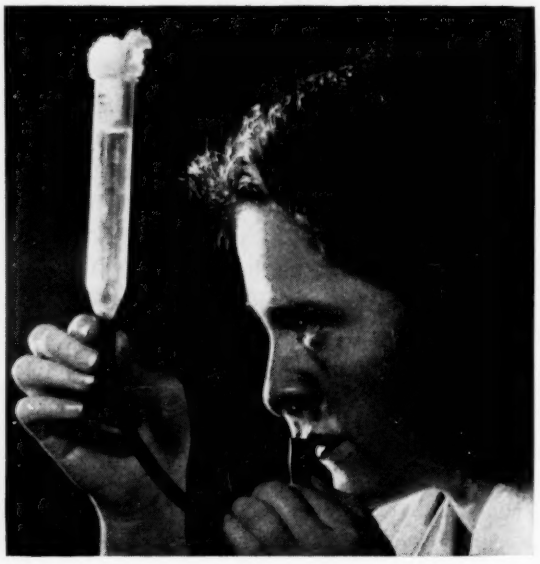

Fig. 2.

Fig. 1.—Sampling device for irrigating the nasal cavity.
Fig. 2.—Showing the position of the sampling device for irrigation of the nasal cavity.

During the following week the antibacterial agent is applied in accordance with the requirements of the experiment and nasal washings are carried out at the same time of day as the preceding week. The next week no intranasal applications are made and washings are continued at the usual time.

RESULTS

To illustrate the degree of variation which may be expected when daily counts are made on healthy subjects without the application of an effective intranasal agent, the following procotol is typical of many of our experiments (Table I).

It will be noted that while there are wide daily variations in the counts, within certain limits the subjects tend to remain in high (Subjects C

TABLE I. NASAL BACTERIA COUNTS ON HEALTHY SUBJECTS; NUMBER OF BACTERIA PER CUBIC CENTIMETER OF NASAL WASHINGS

DATE	SUBJECT				
	A	B	C	D	E
4/29	360	900	9,920	90	26,220
4/30	800	2,000	1,200	330	18,000
5/1	70	860	1,700	400	13,500
5/2	90	260	200	250	10,700
5/3	90	320	2,200	160	1,000
5/6	1,480	520	5,100	400	12,200
5/7	490	630	13,000	110	17,500
5/8	220	1,300	200	120	38,200
5/9	840	1,330	3,000	100	19,800
5/10	530	500	—	300	2,800
Average	497	862	4,058	225	15,992

—, Subject not available.

TABLE II. NASAL BACTERIA COUNTS ON HEALTHY SUBJECTS BEFORE, DURING, AND AFTER PENICILLIN ADMINISTRATION; NUMBER OF BACTERIA PER CUBIC CENTIMETER OF NASAL WASHINGS

	DATE	SUBJECT				
		A	B	E	F	G
Control period	3/5	1,200	590	52,100	185	15,000
	3/6	560	310	—	172	3,600
	3/7	128	400	46,000	360	1,100
	3/8	236	720	35,000	260	600
	Average	531	505	44,366	244	5,075
Penicillin administration	3/11	52	80	240	12	96
	3/12	6	4	350	5	18
	3/13	0	0	324	1	12
	3/14	0	0	250	0	8
	3/15	3	2	195	3	—
	Average	12	17	271	4	33
Control period	3/18	3	26	—	1	47
	3/19	0	48	—	2	—
	3/20	0	210	—	30	480
	3/21	2	65	—	22	68
	3/22	18	400	—	—	1,128
	Average	4	149		14	430

—, Subject not available.

and E), medium (Subjects A and B), or low (Subject D) count groups. Because of this great variation in normal counts, an intranasally applied antibacterial agent must show a very marked reduction in the counts before its effectiveness can be proven statistically. That such a reduction can be achieved is shown by the results in Table II, when the subjects received nose drops containing 500 units of penicillin per cubic centimeter six times daily at hourly intervals and nasal washings were carried out forty to sixty minutes after the last application of penicillin. The washings from three subjects were seeded with a known concentration of penicillin-susceptible *Staphylococcus aureus* organisms and plated in the usual way. These plates did not show any reduction in counts when compared with the control *Staph. aureus* cultures; hence, it is concluded that a carry over of significant amounts of penicillin into the plates did not occur.

There appears to be no doubt that the application of penicillin nose drops produced a marked reduction in the number of nasal bacteria recovered from healthy subjects. In addition, the repressive effect of penicillin seems to persist for a number of days after use. It is interesting to note that Subject E failed to show as low a total count during the penicillin applications as the other subjects. The fact that most of the organisms cultured during this period from the subject were gram-negative, nonsporulating bacilli probably explains this finding.

DISCUSSION

Since the number of bacteria in the nasal cavity probably fluctuates from day to day, the ideal procedure would be to make a control count in the morning, apply the antibacterial agent throughout the day, and make a second count in the late afternoon or evening. However, when this was tried it was found that the morning irrigation alone produced such a marked reduction in the afternoon counts that it was difficult to interpret the effectiveness of an antibacterial agent tested by this procedure. Hence, it appeared necessary to make nasal counts for a week or more before the experiment to obtain an average normal count; then apply the antibacterial agent and compare the counts obtained. As a further check, counts should be made for a week or longer after use of the antibacterial agent is discontinued; if the counts tend to return to the normal level after a reduction during use of the antibacterial agent, it is a strong indication that the agent has been effective. No experiment should be considered satisfactory unless it can be shown that the antibacterial agent is not carried over in the washings in sufficient concentration to exert a bacteriostatic action in the culture plates.

During the trials with five successive washings it was discovered that the first washing removed very few bacteria, while the successive washings removed increasingly more bacteria until a point was reached where further washings showed a progressively diminishing count. Thus, it is essential that each sample consist of at least three separate washings if the most reliable count is to be obtained.

It is believed that the method described in this report for obtaining a quantitative count of the number of bacteria in the nasal cavity offers a reasonably reliable tool in determining the antibacterial action of intranasal agents. It is obvious also that this method may be used for a qualitative as well as a quantitative study of the nasal flora; that is, a differential count of the various types of bacteria may be made when the blood agar plates are examined. The procedure does not pretend to count all of the bacteria in the nasal cavity, since the irrigating solution obviously does not reach all portions of the cavity, but it appears to produce a representative sample of the flora, by washing out approximately the same area of the cavity on repeated irrigations. Variations in counts may be expected due to leakage around the nasal olive and spilling over into the throat, but in our experience subjects soon learn to avoid these losses after a little practice and the nasal counts become correspondingly more reliable.

Finally, it should be emphasized that the reduction in nasal bacteria of healthy subjects by penicillin reported in this paper does not constitute a direct or implied indication that penicillin is of value in the prevention, treatment, or cure of nasal or other respiratory infections.

SUMMARY

A simple sampling device for washing the nasal cavity with saline or broth is described.

A recommended procedure for using the sampling device to determine the number and types of nasal bacteria is outlined.

The effectiveness of penicillin nose drops in reducing the number of nasal bacteria in healthy subjects, as indicated by the results obtained with the sampling device, is described.

REFERENCES

1. Thomson, D., and Thomson, R.: The Common Cold With Special Reference to the Part Played by Streptococci, Pneumococci, and Other Organisms, Ann. Pickett-Thomson Research Lab. **8**: 1-699, 1932.
2. Hamburger, M., Jr., Green, M. J., and Hamburger, V. G.: The problem of the "Dangerous Carrier" of Hemolytic Streptococci. I. Number of Hemolytic Streptococci Expelled by Carriers With Positive and Negative Nose Cultures, J. Infect. Dis. **77**: 68-81, 1945.
3. Hamburger, M., and Lemon, H. M.: The Problem of the Dangerous Carrier of Hemolytic Streptococci. III. The Chemotherapeutic Control of Nasal Carriers, J. A. M. A. **130**: 836-841, 1946.

A MODIFIED TECHNIQUE FOR THE DIFFERENTIATION OF PNEUMOCOCCI FROM ALPHA HEMOLYTIC STREPTOCOCCI USING SODIUM DESOXYCHOLATE

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WITH THE TECHNICAL ASSISTANCE OF KATHERINE P. SIMPSON, B.S.

THE differentiation of the pneumococcus from the alpha hemolytic streptococcus often is impossible on a purely morphologic basis. At present, the chief means of differentiating these two organisms are the time-honored bile solubility test and the results obtained from typing with Neufeld type-specific sera. Today, this latter method often is time consuming because so many patients have had sulfonamide therapy by the time they enter the hospital. Thus, the suspect pneumococci in sputum often will not type unless passed through one or two mice in order to relieve the organisms of the sulfonamide effects. Therefore, in the large percentage of cases, the bile solubility test has offered the most practical means of differentiation.

Since Neufeld's[1] discovery in 1900 that the pneumococcus was soluble in whole bile, many investigators have described various substances and numerous methods by which this organism may be dissolved. By 1907 the pneumococcus was first distinguished by its bile solubility; it was at this time that Nicolle and Adil-Bey[2] recognized that bile salts (sodium cholate and sodium choleate) were more effective than whole bile. Though Dochez and Gillespie[3] apparently found a true bile-insoluble strain of pneumococcus, according to White and associates[4] Mair states: "Different strains of pneumococcus show varying sensitiveness to the action of bile, just as they vary in the readiness with which they undergo autolysis in culture; but with a satisfactory technique one is seldom in doubt as to whether a particular strain should be classed as bile soluble or not, and strains which have been kept on culture media for long periods retain the property." A thorough review of the literature pertinent to this subject is given by White and associates.[4]

Liefson's[5] modification of the bile solubility test proved unsuccessful in our hands. The purpose of this paper is to present what we believe to be a faster and simpler test, which has proved to be accurate. This test, requiring little equipment, is suitable for the laboratory of the intern or for the general hospital.

METHOD

Equipment.—The equipment, as illustrated in Fig. 1, includes the following:

1. Fresh solution of 10 per cent sodium desoxycholate, pH 7.4.
2. Platinum wire loop.

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3. Microscope with oil immersion lens and immersion oil.
4. Bunsen burner.
5. Two glass microscope slides.
6. Distilled water.
7. Gram stain equipment.

Technique.—The test, to be done at a temperature not above 50° C., consists of the following steps:

1. To four drops of fresh 10 per cent sodium desoxycholate on the first glass slide (*I*) add two medium-sized loopsful of the material containing the organism to be tested (washed sputum, broth suspension of the organism, or colonies from agar plate). Allow the slide to stand for ten minutes and shake gently occasionally.

2. On one-half of the second glass slide (*II*) make a direct smear from the original material as a control (*C*).

3. After ten minutes, to one drop of distilled water on the other half of the second glass slide (*II*), add one loopful of material from the mixture of the first glass slide as the test (*T*).

4. Dry this second glass slide (preferably on a warm ring stand), Gram stain, and compare the control smear with the test smear under oil immersion. The absence on the test smear of gram-positive encapsulated organisms, such as are present on the control smear, indicates a positive test. The presence of such organisms on both test and control smears indicates a negative test; in other words, the organisms tested are not pneumococci.

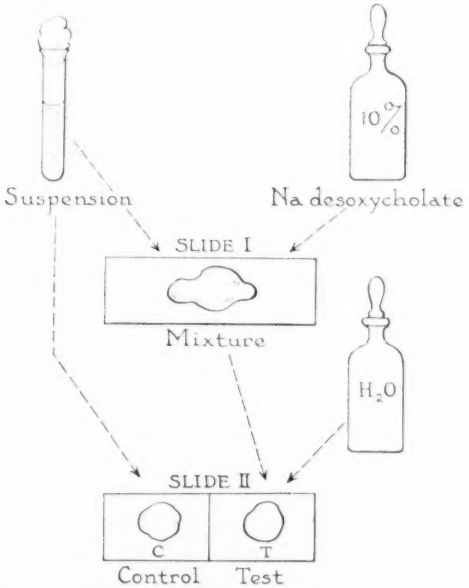

Fig. 1.—Diagram of procedure.

RESULTS

This method was used in testing six different types of pneumococci previously identified by serologic typing and one strain of gamma nonhemolytic streptococcus. In Fig. 2 the results obtained by our method are compared with those obtained by Liefson's method in fifteen cases. In one of these cases there

was only partial solution of the pneumococci with our method. Using Liefson's technique we found complete solubility in one case and no appreciable solubility in the others.

As a means of checking our technique, we compared its results with those obtained by the standard macroscopic method described by Kolmer and Boerner[6] in a series of twenty-five different types of pneumococci in pure culture. Complete solubility occurred by both methods in all the types tested.

Repeated success with our method during the year in a series of over 200 cases, and covering more than 500 separate tests, constitutes further indication of its accuracy. In no instance in seventy-seven cases of known streptococcal infections tested have we ever dissolved the alpha, beta, or gamma streptococci by this method.

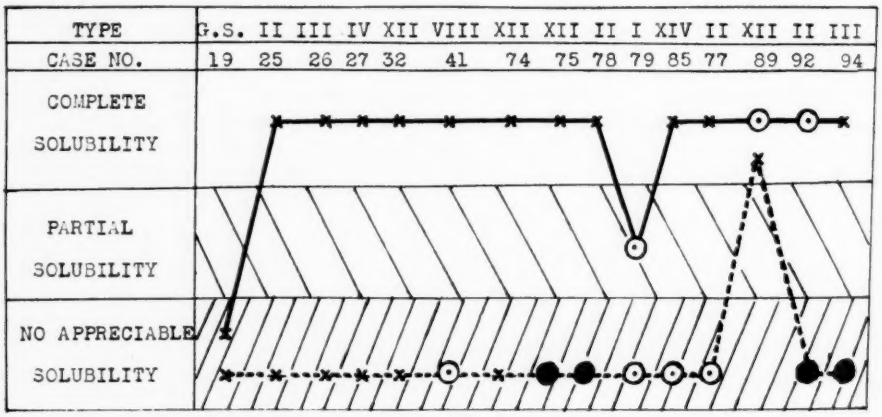
TYPE
G.S. II III IV XII VIII XII XII II I XIV II XII II III
CASE NO.
19 25 26 27 32 41 74 75 78 79 85 77 89 92 94
COMPLETE SOLUBILITY
PARTIAL SOLUBILITY
NO APPRECIABLE SOLUBILITY

Fig. 2.— - - - -, Liefson's method. ——, Slide method. ⊙, "Quellung shadows" observed with the addition of type-specific serum. ●, "Quellung shadows" and also ordinary "Quellung phenomenon" observed with the addition of type-specific serum. G. S., Gamma streptococcus; roman numerals refer to pneumococcus types.

In the course of this work the observation was made that if the test (*T*) was not allowed to dry, but instead type-specific serum was added directly to the moist preparation, the presence of enlarged capsules or "Quellung shadows" were noted eight times in Liefson's test and three times in our test in the representative series (Fig. 2). The bodies of the organisms were apparently dissolved by the bile salt and the capsules remained intact. The intact capsules were invisible until type-specific serum was used and the test was examined under oil immersion with a dull light; the capsules appeared translucent and took no stain, whereas the body, or normally staining portion, was absent.

COMMENT

In a study of twenty-seven pneumococcal infections, 77 streptococcal infections, and 11 miscellaneous infections, the slide test described in this report served as an accurate method of identifying pneumococci in spinal fluid, washed sputum, and in either pure or mixed cultures grown on blood or protease agar media. In the latter instance, when the contaminating organisms were alpha

hemolytic streptococci, we found that the addition of the six groups of Neufeld typing sera served to identify the pneumococci by "Quellung shadows" and the alpha hemolytic streptococci as undissolved intact organisms.

The presence of the "Quellung shadows" suggests that proteolysis occurs in the presence of excess sodium desoxycholate. Goebel and Avery[7] have shown that autolysis is accompanied by proteolysis and lipolysis, whereas sodium desoxycholate, in excess, inhibits the action of pneumococcal protease, but not lipase. These authors, however, found that pneumococcus is dissolved in the presence of an excess of the bile salt and concluded that bile solubility is probably not identical with autolysis. We also found that the body of the organism is dissolved even though the protease is apparently inhibited in the presence of excess sodium desoxycholate. Further, we found an apparent resistance at times of the carbohydrate or capsular portion of the pneumococcus to the action of excess sodium desoxycholate as was clearly shown by the "Quellung shadow" phenomenon.

SUMMARY

1. A simple modification of the bile solubility test is described.

2. The presence of the "Quellung shadows" in bile-tested material from mixed cultures is suggested as a further aid in the differentiation between pneumococci and alpha hemolytic streptococci.

3. The significance of the "Quellung shadows" is discussed.

REFERENCES

1. Neufeld, F.: Ueber eine specifsche bakteriolytische Wirkung der Galle, Ztschr. f. Hyg. u. Infectionskrankh. **34**: 454, 1900.
2. Nicolle, M., and Adil-Bey: Action de le bile sur le Pneumocoque, et diverse autres bacterier. Ann. de l'Inst Pasteur **21** (1): 20, 1907.
3. Dochez, A. R., and Gillespie, L. J.: A Biologic Classification of Pneumococci by Means of Immunity Reactions, J. A. M. A. **61**: (10): 727, 1913.
4. White, B., Robinson, E. S., and Barnes, L. A.: The Biology of Pneumococcus, Commonwealth Fund, London, 1938, Oxford University Press, pp. 55-62.
5. Liefson, E.: Use of Sodium Desoxycholate for Identification of Pneumococci, J. A. M. A. **104**: 213, 1935.
6. Kolmer, J. A., and Boerner, F.: Approved Laboratory Technic, ed. 4, New York, 1945, D. Appleton-Century Company, Inc., pp. 392-393.
7. Goebel, W. F., and Avery, O. T.: A Study of Pneumococcus Autolysis, J. Exper. Med. **49** (2): 267, 1929.

A SIMPLE APPARATUS FOR CULTURING NEISSERIA GONORRHEA UNDER PARTIAL CARBON DIOXIDE TENSION

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AN EVER-INCREASING demand for the isolation and identification of *Neisseria gonorrhea* by culture methods, not only as an aid to diagnosis, but also as a criterion for cure of both male and female patients following chemotherapy, makes it desirable that a simple apparatus for culturing gonococci be made available.*

The following simple apparatus has been devised to meet this demand and has proved to be readily applicable for routine gonococcus culture methods.

The apparatus consists of a standard laboratory desiccator (Scheibler type, 150 mm.), having an approximate capacity of 2,500 c.c. and a plexiglass Petri dish holder, so constructed to accommodate either a medicine dosage glass or a porcelain crucible as a reaction vessel for the production of carbon dioxide by the action of dilute HCl with sodium bicarbonate.

The Petri dish holder (Fig. 1) is constructed from the ordinary plastic, plexiglass (¼ inch thickness), and the component parts of the holder (top, base, and three supports) are cemented together with an adhesive made by dissolving plastic shavings in acetone. Increased rigidity of the culture plate holder is accomplished by dovetailing the supports at the top and base. Since the culture plate holder fits snugly in the desiccator, a series of holes (0.4 cm.) are drilled in the top and base plates to insure adequate circulation of CO_2 throughout the desiccator. Sufficient CO_2 tension is produced by the addition of 3 or 4 c.c. of 10 per cent HCl to approximately 1 Gm. of sodium bicarbonate.

The following procedure is employed in preparing the apparatus for incubation. After the culture plates have been inoculated, they are placed in the culture plate holder, which is then introduced into the desiccator. The reaction vessel containing the sodium bicarbonate (Fig. 2, *A*) is placed in the round opening on the top plate of the culture dish holder. The lid of the desiccator is tightly sealed in place, leaving the stopcock open. Dilute HCl is then delivered with a 5 c.c. diluting pipette through the open stopcock into the reacting vessel. The stopcock is immediately closed and the entire apparatus is placed in a bacteriologic incubator for the desired length of incubation at 37° centrigrade.

The advantages of an all-glass culture apparatus, plus the noncorrosive plexiglass culture plate holder, are obvious when one remembers the disagreeable features of using a copper Petri dish holder utilizing a burning candle for the production of carbon dioxide. The previously described apparatus can easily be cleaned and sterilized after each incubation period. If additional moisture is

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*The apparatus was devised and used by the author while serving as technical sergeant, Medical Department, in the Armed Forces of the United States.

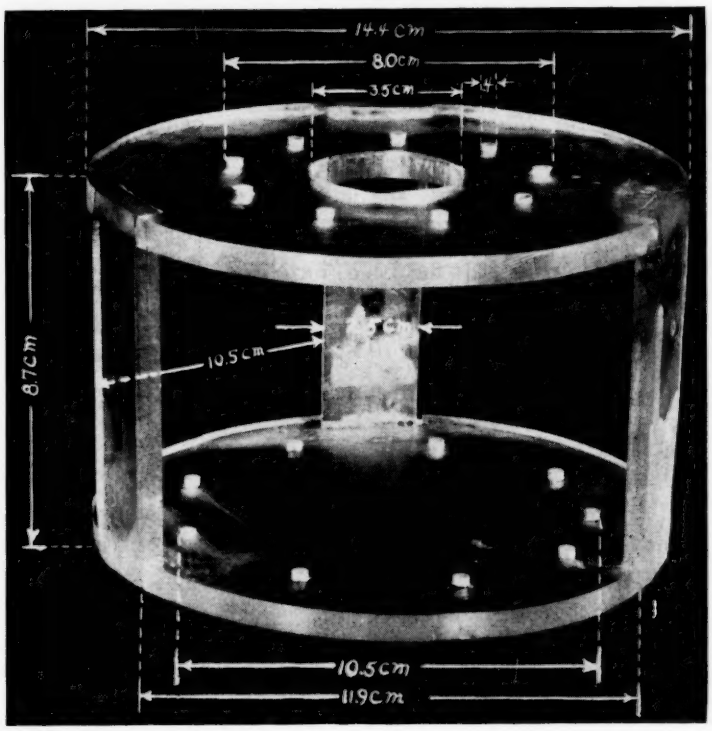
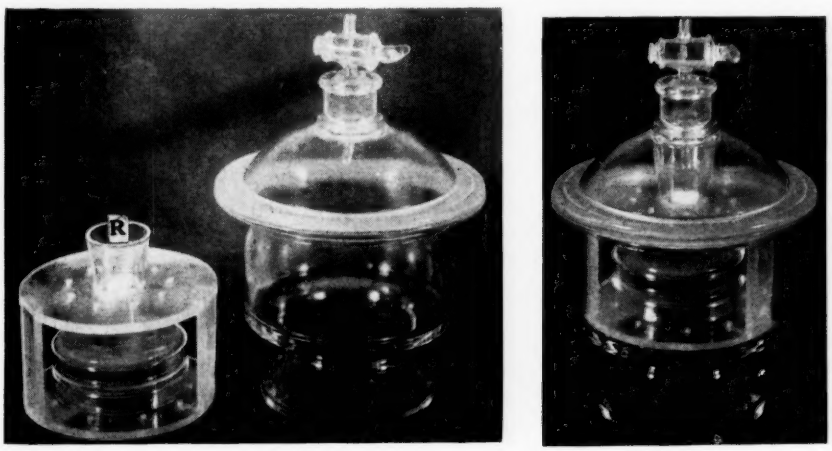


Fig. 1.—Plexiglass Petri dish holder, with a 3.5 cm. hole in the top plate to accommodate either a medicine dosage glass or porcelain crucible as a reaction vessel for the production of carbon dioxide.



A. *B.*

Fig. 2.—*A*, View of the culture apparatus, showing the reaction chamber (*R*) and inoculated culture plates ready for introduction into the desiccator. *B*, Assembled gonococcus culture apparatus ready for incubation.

necessary to safeguard against dehydration of the culture media during incubation, a very small amount of water can be placed on the floor of the desiccator before the culture plate holder is introduced into the desiccator.

The apparatus has been used by the author for more than one year and found satisfactory for routine gonococci cultures, using either chocolate-agar (Difco) or Peizer media.

SUMMARY

A simple apparatus for culturing *Neisseria gonorrhea* under partial carbon dioxide tension is described. It has been found to be adequate for the culture of gonococci as an aid to diagnosis.

A TEST FLUID FOR CONTROL OF THE GOLD REACTION

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CORRECT results in the colloidal gold test depend primarily upon two physicochemical factors[1]: the first is the pH of the milieu,[2] which is optimal at 7.4, and the second is the sensitivity (dispersity) of the gold sol, which should be controlled by use of a photoelectric colorimeter.[3] A test fluid is required as an additional safeguard to demonstrate that optimal conditions have been secured.

Such a test fluid may be prepared by pooling approximately equal amounts of twenty or more normal sera. Specimens that are chylous or otherwise abnormal in appearance, or that react in the complement-fixation test for syphilis, are excluded. Additional criteria for the "normality" of the sera are not used. Exactly 5 ml. of the pooled sera are diluted with phosphate buffer, pH 7.4,[4] in a 250 ml. volumetric flask. Fifty milliliters of this solution are diluted with 50 ml. of glycerol, the final dilution being 1:100. The fluid is kept in the refrigerator and is freshly prepared each month. One-tenth milliliter is tested in duplicate every working day in the same manner as a specimen of cerebrospinal fluid.

The test fluid when satisfactory yields a uniformly standard curve characterized by three factors: (1) The degree of coagulation, determined by the gold color standard, is 6 in the first or 1:15 dilution; (2) the maximum reaction, 15, occurs in dilution 1:114; and (3) the sum of the ten numerical color values is between 95 and 100. The exact reproducibility of the test fluid was demonstrated by testing ten different serum pools at one time with the same reagents. The results were identical with reference to the three factors mentioned. The negligible deviations in other dilutions, particularly in the postzone, are not due to differences of the test fluid but to inevitable slight inaccuracies of pipetting. To obtain such results, the dilution of the serum pool (1:100) must be exact. Experience has shown that on rare occasions an abnormal serum that has been inadvertently included may spoil a pool.

The standard curve is the final criterion of the suitability of the test fluid and other reagents and particularly of the gold color standard. In fact, it provides a rigorous control of the test as a whole. Deviations from the standard curve indicate that a reagent is unsatisfactory. The source of the deviation is determined by replacing one reagent at a time, which can be done easily if a set of reagents known to be satisfactory is available. For example, if a new buffer is substituted and the test fluid does not give the standard curve, the new buffer solution is evidently at fault.

Use of the test fluid is illustrated in Table I. In the first four items, in which the colloidal gold solutions are of satisfactory sensitivity as indicated by the turbidimetric value, the pH of the milieu is 7.4 by potentiometric determination and the buffer is of satisfactory pH and molar concentration. The

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TABLE I. COLLOIDAL GOLD REACTIONS WITH TEST FLUID PREPARED FROM POOL OF NORMAL SERA

SERIAL NUMBER	COLLOIDAL GOLD SOLUTIONS* (DATE OF PREPARATION)	TURBIDITY	TEST FLUID PREPARED	pH OF MILIEU†	MOLARITY	COLLOIDAL GOLD REACTIONS NEW SERIAL DILUTIONS 1:15	23	34	51	76	114	171	256	384	576	SUM TOTAL	
1	6/25/46	135	6/13/46	7.4	M/21.5	6	7.5	8.5	12	13	15‡	14	9	7	6.5	99	Standard curve; every reagent correct
2	6/ 6/46	122	6/13/46	7.4		6	7	9	11	13	15	14	8.5	6	5.5	95	Standard curve; every reagent correct
3	2/28/46	128	2/18/46	7.4		6	7.5	9.5	13	14	15	14	9	6.5	5	100	Standard curve; every reagent correct
4	2/28/46	128	3/ 4/46	7.4		6	8	9.5	13	14	15	14	10	7	3	100	Standard curve; every reagent correct
5	6/25/46	135	6/13/46	5.2	M/20	9	13	15	16	18	18	18	18	16	16	157	Inadequate pH of milieu
6	6/25/46	135	6/13/46	6.0	M/20	8	9	12	14	14	9.5	9	6	5	4	91	Inadequate pH of milieu
7	6/25/46	135	6/13/46	6.4	M/20	7	8	10	13	14	14	9	7	5.5	4	92	Inadequate pH of milieu
8	6/25/46	135	6/13/46	7.4	M/ 5.4	8.5	15	18	18	18	18	18	18	18	18	168	Inadequate electrolyte concentration
9	3/27/46	73	3/ 4/46	7.4	M/21.5	4.5	6.5	9.5	13	14	15	14	9.5	7	4.5	98	Inadequate sensitivity of gold sol
10	12/13/46	70	10/22/46	7.4	M/21.5	3.5	6	9	10	13	11	7	6	4.5	3	73	Inadequate sensitivity of gold sol

*Citrate gold 1:10,000[3] was used.

†The routine phosphate buffer pH 7.4 was used except in the case of Nos. 5, 6, and 7 in which phthalate buffer pH 5.2, pH 6.0, and pH 6.4, respectively, were used.

‡The ^ indicates the location of the maximum reaction in prezone curves.

findings obtained are almost identical, although the gold sols were prepared on different days and different test fluids were used. When the pH of the milieu is more acid than 7.4, the results vary markedly from those obtained when the pH is maintained at 7.4, as in items 5, 6, and 7. When the molar concentration of the buffer is incorrect (item 8), the results again show clearly that a mistake has been made. The pH and the molar concentration are controlled simultaneously by the test fluid, which is not possible by potentiometric determination.

In items 9 and 10, citrate gold sols 1:10,000 prepared with impure distilled water were used. The turbidity and sensitivity of these sols were abnormally low. Below a certain limit, abnormality is clearly demonstrable by the test fluid. In both Nos. 9 and 10, the degree of coagulation in the prezone is abnormal; in No. 10, even the location of the maximum is shifted and the sum total is markedly decreased.

Two controls for particle size and reproducibility of the test as a whole (turbidimetric determinations and use of a test fluid) are recommended. A gold sol having an abnormal particle size can be detected by turbidimetry. Such findings indicate the use of impure distilled water. The test fluid, however, shows in detail the type and degree of deviation from the standard reaction.

OTHER TEST FLUIDS

Before pools of normal sera were found to provide a satisfactory test fluid, the value of other types of fluid was studied as follows:

1. Four cerebrospinal fluids of different protein patterns (types A, B, C, and D), preserved by adding equal amounts of glycerol, were used. If the pH and the sensitivity were correctly adjusted, reproducible results were obtained. It was difficult, however, to obtain four suitable fluids for the purpose, and when a satisfactory specimen was obtained, only a small amount was available.

2. Use of cerebrospinal fluids from patients with paresis[6] is unsatisfactory. Only test fluids yielding a prezone curve, such as types B or C,* can demonstrate that a false D (paretic) curve[2] has been obtained if the pH is too acid.

3. Purified globulin fractions yield either plateau (D) or prezone (B or C) curves. Even if the pH of the milieu is maintained at 7.4, test fluids giving a plateau (paretic or type D) curve are inadequate. A solution of 20 mg. per 100 ml. of gamma globulin was found to yield the same plateau or first-zone curve as a specimen of cerebrospinal fluid from a paretic. A plateau of complete coagulation was obtained with both, up to a final dilution of 1:320. A solution of 10 mg. per 100 ml. of globulin gave a plateau of complete coagulation in a 1:160 dilution. A solution of 15 mg. per 100 ml., however, could not be differentiated by end-point titration from a solution of either 10 or 20 mg. per 100 ml. Hence, test fluids that yield a plateau curve, besides being inadequate as a control of the pH, provide only a rough control in the quantitative adjustment or sensitivity.

*Citrate gold 1:10,000 was used.
†The routine phosphate buffer pH 7.4 was used except in the case of Nos. 5, 6, and 7 in which pH 6.4, respectively, were used.
‡The ^ indicates the location of the maximum reaction in prezone curves.

*Classification of curves obtained in the colloidal gold test: A, Obtained with normal cerebrospinal fluid; B, formerly designated as second zone or syphilitic; C, formerly designated as hematogenous, third zone, or meningitic; D, formerly designated as paretic or first-zone curve.

4. Edestin,[7] widely used as a test fluid, yields a first-zone curve and in some respects is even inferior to gamma globulin.

5. Pseudoglobulin obtained from normal horse serum by repeated fractionation, between 33 and 45 per cent saturation with ammonium sulfate, produces a prezone curve. It provides a highly satisfactory control on quantitative and qualitative reproducibility of the gold reaction. Its preparation is somewhat complicated, however, and the reproducibility of the test fluid itself is unsatisfactory.

SUMMARY

A test fluid prepared from pools of normal sera for use as a control in the colloidal gold test is described. The fluid is simple to prepare and reproducible. The procedure recommended permits identical results to be obtained in different laboratories and is a step forward in standardization of the gold reaction.

REFERENCES

1. Lange, C.: Theory of the Colloidal Gold Reaction, J. Lab. & Clin. Med. **30**: 1006-1012, 1945.
2. Lange, C., and Harris, A. H.: The Significance of the pH in the Gold Reaction, J. Lab. & Clin. Med. **29**: 970-975, 1944.
3. Lange, C., and Harris, A. H.: A Citrate Gold of Optimal and Reproducible Sensitivity for Use in the Colloidal Gold Reaction, Am. J. Pub. Health. **34**: 1087-1092, 1944.
4. Lange, C.: Methods for the Examination of Spinal Fluid, Am. J. Syph., Gonor. & Ven. Dis. **23**: 638-668, 1939.
5. Lange, C.: Die Ausflockung kolloidalen Goldes durch Zerebrospinal flüssigkeit bei luetischen Affektionen des Zentralnervensystems, Ztschr. f. Chemotherap. **1**: 44-78, 1912.
6. Adams, D. K., and Scott, W. H.: The Colloidal Gold Reaction of Cerebrospinal Fluid; Standardising and Application in Early Nervous Disease, J. Path. & Bact. (Proc. Soc.) **25**: 142-143, 1922.
7. Kreidler, W. A., and Small, J. C.: A Method of Standardizing Colloidal Gold Sols by Utilizing a Standard Solution of Globulin, J. Lab. & Clin. Med. **17**: 269-271, 1931.